



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 1/21, A61K 39/106	A1	(11) International Publication Number: WO 94/01533 (43) International Publication Date: 20 January 1994 (20.01.94)
(21) International Application Number: PCT/US93/06270 (22) International Filing Date: 1 July 1993 (01.07.93) (30) Priority data: 07/909,382 6 July 1992 (06.07.92) US (71) Applicants: PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 17 Quincy Street, Cambridge, MA 02139 (US). VIRUS RESEARCH INSTITUTE [US/US]; 61 Moulton Street, Cambridge, MA 02138 (US). (72) Inventors: MEKALANOS, John, J. ; 78 Fresh Pond Lane, Cambridge, MA 02138 (US). BEATTIE, David ; 10 Neponset Court, Boston, MA 02131 (US). KILLEEN, Kevin ; 1112 Brook Road, Milton, MA 02186 (US). LU, Yichen ; 15 South Woodside Avenue, Wellesley, MA 02181 (US).	(74) Agent: FREEMAN, John, W.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110-2804 (US). (81) Designated States: AU, BB, BG, BR, CA, CZ, FI, HU, JP, KP, KR, LK, MN, NO, NZ, PL, RO, RU, SD, SK, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	
(54) Title: DELETION MUTANTS AS VACCINES FOR CHOLERA (57) Abstract The invention features of nontoxigenic, genetically stable mutant strains of <i>V. cholerae</i> and a method of making which are useful as live, oral vaccines for inducing immunological protection against cholera. The mutant strains are genetically engineered mutants which lack DNA encoding a functional ctxA subunit which is responsible for many of the symptoms of cholera. The strains also lack any functional attRS1 sequences which are required for recombination and amplification of the CTX genetic element. These strains are safe because they can not recombine with the wild type attRS1-containing vehicles which include the ctxA-encoding DNA.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LJ	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LU	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TG	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

- 1 -

DELETION MUTANTS AS VACCINES FOR CHOLERABackground of the Invention

The field of invention is *Vibrio cholerae* vaccines. After more than 100 years of research on cholera, there remains a need for an effective cholera vaccine. There have been six pandemics of this disease caused by strains of *V. cholera* belonging to the "Classical" biotype. The etiological agents of the current (seventh) pandemic belong to the "El Tor" biotype (Finkelstein, Crit. Rev. Microbiol 2:553-623, 1973, Wachsmuth et al., The Lancet 337:1097-1098, 1991). Recently the seventh pandemic has extended to a new locale, that of South America. Beginning in January of 1991, an epidemic of cholera resulted in greater than 250,000 cases and over 2,000 deaths in Peru, Ecuador, Columbia, and Chile. Before this epidemic it was estimated that over 200,000 cases of cholera occurred per year mainly in India, Bangladesh, Africa and Western Asia (Tacket et al., Cholera Vaccines. In Vaccines: New Approaches to Immunological Problems, Ellis, R. W., editor, Butterworth-Heinemann, Boston, 1992).

In November of 1992, an antigenically distinct, non-01 form of *V. cholerae* emerged in India and Bangladesh and within eight months caused an estimated 500,000 cases and 6,000 deaths. The pandemic potential of this new strain, designated serogroup 0139 synonym "Bengal", seems assured and is a new cause of concern throughout the developing world. These recent experiences underline the need for effective cholera vaccines against disease due to both El Tor 01 and Bengal 0139 serotypes of *V. cholerae*.

- 2 -

Because natural infection by and recovery from cholera induces immunity lasting at least 3 years (Tacket et al., *Supra*; Levine et al., J. Infect. Dis. 143:818-820, 1981; Cash et al., J. Infect. Dis. 130:325-333, 5 1974), much effort has been made to produce live, attenuated cholera vaccines that when administered orally would mimic the disease in its immunization properties but would not cause adverse symptoms or reactions in the immunized individual (i. e., display low reactogenicity).

10 Vaccines of this type involve deletion mutations that inactivate the gene encoding the A subunit of cholera toxin, a protein which is responsible for most of the diarrhea seen in this disease (Mekalanos et al., Proc. Natl. Acad. Sci. USA 79:151-155, 1982; Mekalanos et al., 15 Nature 306:551-557, 1983; Kaper et al., Nature 308:655-658, 1984; Kaper et al., Biotechnology 2:345, 1984; Pierce et al., Infect. Immun. 55:477-481, 1987; Taylor et al., Vaccine 6:151-154, 1988; Levine et al., Infn. Immun. 56: 161-167, 1988; Herrington et al. J. Exper. 20 Med. 168:1487-1492, 1988; Levine et al., Lancet ii:467-470, 1988; Kaper et al., Res. Microbiol. 141:901-906, 1990; Pearson et al., Res. Microbiol. 141:893-899, 1990). See also Mekalanos, U.S. Patent Nos. 5,098,998 and 4,882,278, and Kaper et al., U.S. Patent No. 25 4,935,364, hereby incorporated by reference. While both oral, killed whole cell vaccines and several live, attenuated cholera vaccine have been developed, the most promising of these provide little protection against the El Tor biotype of *V. cholerae* and probably no protection 30 against the 0139 serotype. The major issues associated with cholera vaccines are safety, stability and their degree of antigenicity.

With regard to the toxin genes of *V. cholerae*, the genetic diversity among toxigenic and non-toxigenic 35 strains has been examined by Chen et al. (1991,

- 3 -

Epidemiol. Infect. 107:225). Mekalanos (1983, Cell 35:253) reports on the duplication and amplification of *V. cholerae* toxin genes, and Miller et al. (1984, Proc. Natl. Acad. Sci. USA 81:3471) discusses transcriptional
5 regulation of the toxin genes. Other *V. cholerae* genes whose products may play a role in the pathogenicity of this organism include the toxin-coregulated pilus genes (Shaw et al., 1990, Infect. Immun. 58:3042; Sharma et al., 1989, Vaccine, 7:451; Sun et al., 1990, J. Infect.
10 Dis. 161:1231; Hall et al., 1991, Infect. Immun. 59:2508; Taylor et al., 1987, Proc. Natl. Acad. Sci. USA 84:2833), and the gene encoding the intestinal colonalization factor (Taylor et al., 1988, Vaccine 6:151).

Summary of the Invention

15 The invention features a nontoxigenic genetically stable mutant strains of *V. cholerae* which are useful as a live, oral vaccines for inducing immunological protection against cholera. The mutant strains are genetically engineered mutants which lack DNA encoding a
20 functional *ctxA* subunit and also lack any functional *attRS1* sequences. By *attRS1* sequences is meant a 17 base pair sequence contained within the CTX genetic element that is required for recombination and amplification of the CTX genetic element, or enough of that sequence to
25 enable such recombination and amplification. Mutants which "lack any functional *attRS1* sequences" are those which substantially cannot undergo effective site-specific recombination with *attRS1*-containing vehicles, because the wild type *attRS1* sequences are wholly deleted
30 or are sufficiently deleted or mutated to prevent such recombination. As a result, *V. cholerae* strains according to the invention are safer because they cannot recombine with wild type *attRS1*-containing vehicles which include the *ctxA*-encoding DNA.

- 4 -

The invention also features a method of making the above described *V. cholerae* strains. The method involves introducing a plasmid into a wild type *V. cholerae* which contains a fragment of *V. cholerae* DNA containing a
5 mutation in the *ctxA* and *attRS1* sequences. The *V. cholerae* DNA fragment is capable of recombining with wild type *V. cholerae* DNA inside the organism to generate the mutant strain.

Although any serotype of *V. cholerae* may be used,
10 in preferred embodiments, the mutant strain of *V. cholerae* belongs to the El Tor serotype, and more preferably, the Inaba or Ogawa serotype or the *V. cholerae* non-01 serotype, preferably 0139 "Bengal" serotype. Preferably, the mutants lack all of the CTX
15 core and *attRS1* sequences and more preferably the mutant strain is Peru-2, Bang-2, Bah-2, or an attenuated derivative of the Bengal serotype, such as Bengal-2 ("Beng-2") or Bengal-3 ("Beng-3") as described below.

Mutant strains according to the invention
20 optionally include additional mutations introduced to improve the safety and/or the immunogenicity of the vaccine. Such additional mutations include, but are not limited to, inactivation of one or more genes involved in DNA recombination, for example the *recA* gene encoded by
25 the strain, and the introduction of additional genes which may be introduced into the *V. cholerae* chromosome, preferably into the *V. cholerae lacZ* gene. Preferred additional genes include a gene encoding the B subunit of *V. cholerae* or any heterologous antigen such as the B
30 subunit of Shiga-like toxin, or a gene encoding the *E.coli* CFA antigen, or an antigenic HIV antigen. By heterologous antigen is meant any antigen that is not normally expressed by *V. cholerae*. For example, the heterologous antigen may be *Shigella* lipopolysaccharide
35 (LPS) antigen, Shiga-toxin, various CFA antigens of

- 5 -

enterotoxigenic *E. coli* strains, anthrax toxin, Pseudomonas endotoxin A, antigenic fragments from the HIV capsid, pertussis toxin, tetanus toxin; antigens from Herpes virus, rubella virus, influenza virus, mumps virus, measles virus, poliomyelitis virus; and immunogenic polypeptides from eukaryotic parasites causing malaria, pneumocystis pneumonia, and toxoplasmosis, may be expressed in a *V. cholerae* live vaccine. Preferably, the mutant strain having additional mutations is Peru-14, Peru-3, Peru-4, Peru-5, Bang-3, Bang-5, Bah-3, Bah-4, Bah-5 or an attenuated derivative of Bengal.

By a ctxA subunit is meant the A subunit of the cholera toxin which is responsible, when functional, for many of the symptoms of cholera (e.g., nausea, diarrhea etc.). Most preferably, the strains include deletion of the entire so-called "core genetic element", includes not only the ctxA/B, but also a region known as ICF (Intestinal Colonization Factor, probably equivalent CEP "core encoded pilin") and ZOT, described in greater detail below.

In another aspect, the invention features a nontoxigenic genetically stable mutant strain of *V. cholerae* which is useful as a live, oral vaccine for inducing immunological protection against cholera. The mutant strain is a genetically engineered mutant which lacks DNA encoding a functional ctxA subunit. The strain may also be soft agar penetration-defective. By soft agar penetration-defective is meant lacking the ability to penetrate a media of high viscosity as measured *in vitro* by swarming on and within agar media which is between 0.25 and 0.4% agar. The preferable strain may also be filamentous, i.e. 25% or more cells greater than 15 μ m in length under conditions of logarithmic growth. In preferred embodiments the strain is also ATT-.

- 6 -

In preferred embodiments, the invention includes a vaccine comprising at least two different strains of *V. cholerae* which are nontoxigenic genetically stable mutants which lack DNA encoding a functional ctxA subunit
5 and are also soft agar penetration-defective. One of the two strains is preferably derived from the Peru strain and the other one is derived from the Bengal strain. The invention also includes a vaccine in which each of the component strains are ctx⁻, att⁻, and recA⁻. Depending
10 upon the relevant local epidemiology, the vaccine strains may be administered together in a single dose, or more preferably, separately 7-28 days apart. Where only one of the serotypes presents a threat of disease, it may be preferable to administer a vaccine regime comprising only
15 one strain.

The invention also features a killed, oral cholera vaccine comprising at least a first and a second *V. cholerae* strain, wherein at least two of the strains are different serotypes and all strains in the mixture lack
20 DNA encoding a functional ctxA subunit. The vaccine also contains cholera toxin B subunit produced by at least one of the serotypes. Preferably, one of the serotypes in the vaccine is an Ogawa serotype and another of the serotypes is an Inaba serotype. Most preferably, the
25 killed oral vaccine comprises Bah-3 and either Peru-3 or Bang-3, or both Peru-3 and Bang-3, as defined below. Any of the oral vaccine combinations may also include cells of the Bengal serotype, as defined below, including Bengal-2 and Bengal-3. The strains may be administered
30 singly, together, or in consecutive doses 7-28 days apart.

The invention also features a method of making a killed *V. cholerae* vaccine. The method involves growing at least a first and a second *V. cholerae* strain, wherein
35 each strain in the mixture lacks DNA encoding a

- 7 -

functional ctxA subunit. The strains are then collected from the growth medium and the cells are killed. Cholera toxin B subunit, produced by at least one of the strains is obtained from the medium in which the strain was
5 propagated and is added to the killed cells. The mixture of killed bacteria and cholera toxin B subunit is then suspended in a physiologically acceptable carrier.

Mutants such as those described above are useful as cholera vaccines and are improved in their genetic
10 properties compared with previous vaccines.

Other features and advantages of the invention will be apparent from the following description of preferred embodiments thereof, and from the claims.

Detailed Description

15 The drawings will first be briefly described.

The Drawings

Fig. 1. is a schematic diagram of the CTX genetic elements of toxigenic *V. cholerae* strains P27459-Sm, C6709-Sm and E7946-Sm. The filled in boxes represent RS1
20 sequences. Between the RS1 sequences is a region shown as an open box (called the core region) which contains the ctxAB genes and genes encoding zot, the intestinal colonization factor (ICF). At the ends of the RS1 sequences are filled in circles that represent copies of
25 sequences that match 16 out of 17 bases with the 17 base pair sequence attRS1 (CCTAGTGCGCATTATGT) [SEQ.ID.NO:1]. Although the CTX elements of the three strains vary in their structure based on the number of copies of the RS1 and core regions, it should be noted that these elements
30 are inserted into the same chromosomal site in all El Tor strains of *V. cholerae*.

Fig. 2. (A) Restriction map of the chromosome containing the CTX region from strain C6709-Sm with the CTX element schematically shown as in Fig. 1. Not shown

- 8 -

are the restriction maps of strain P27459-Sm and E7946-Sm which are the same except for the variation observed in sites that map within the CTX element's core or RS1 sequences as designated schematically in Fig. 1. (B)

- 5 Restriction map of corresponding chromosomal region of strain Bang-1, Bah-1, and Peru-1.

Fig. 3. (A) Restriction map of plasmid pGP60 that carries an inserted DNA fragment corresponding to the chromosome containing the CTX region from strain
10 P27459-Sm with the CTX element schematically shown as in Fig. 1. Below this is a two headed arrow which designates the DNA which has been deleted in plasmid pAR62. (B) The restriction map of the CTX region of strain P27459-Sm is shown including restriction sites
15 that map outside the region cloned on plasmid pGP60. (C) A demonstration of the recombinational events (broken lines) between plasmid pAR62 and the chromosome that produced the Type-2 deletion which gave rise in parental strains C6709-Sm, P27459-Sm and E7946-Sm to deletion
20 mutants Peru-2, Bang-2, and Bah-2, respectively. (D) Restriction map of the chromosome of strains Peru-2, Bang-2, and Bah-2.

Fig. 4 is a diagrammatical representation of the construction of plasmid pGP52.

- 25 Fig. 5 is a diagrammatical representation of the generation of pJM84.1 and pJM84.2. A 0.6 kb fragment encoding a promoterless B-subunit was generated by PCR. This DNA was ligated into pCR100 and digested with SpeI/EcoRI. The resulting 0.6 kb restriction fragment
30 was ligated into EcoRI/XbaI digested pVC100 and pRT41 vectors, yielding pJM1001 and pJM411, respectively. Each plasmid was digested with BamHI/EcoRI, treated with Klenow, flanked with XbaI linkers, and digested with XbaI. Purified fragments were ligated to XbaI digested
35 pGP84, yielding pJM84.1 and pJM84.2.

- 9 -

Fig. 6 is a diagrammatical representation of the insertion of the *ctxB* into the chromosome. Non-replicative pJM84.1 was integrated into Peru-2, Bang-2 or Bah-2 by homologous recombination. Ampicillin resistant
5 recombinant colonies were subsequently plated on medium which contained streptomycin without ampicillin, thus reducing the selective pressure for ampicillin resistance. The resulting ampicillin sensitive colonies were isolated and had selected for excision of DNA
10 flanked by homologous *recA* DNA sequences.

The invention features attenuated strains of *V. cholerae* that can be used either as live or killed oral vaccines to protect individuals against cholera and potentially other diseases.

15 Construction of Vaccines

Attenuated derivatives of a *V. cholerae* strain C6709-Sm isolated from a cholera patient in Peru in 1991 have been constructed that can be used as live, oral cholera vaccines. The derivatives Peru-1 and Peru-2,
20 carry small Type-1 (core) and large Type-2 deletions, respectively, which remove the DNA encoding the cholera toxin in addition to DNA encoding *zot*, an intestinal colonization factor (ICF) that is unrelated to cholera toxin. Because excessive intestinal colonization may be
25 responsible for adverse side effects seen in humans administered earlier prototype live cholera vaccines, the deletion of genes encoding both cholera toxin and ICF in Peru-1 and Peru-2 will render these strains less reactogenic in vaccinees while they retain their
30 immunogenic and therefore protective properties.

The larger Type-2 deletion present in Peru-2 also removes an insertion-like sequence called RS1 which is present in two or more copies as part of a larger DNA

- 10 -

segment called the CTX genetic element. The RS1 sequence encodes a site-specific recombination system that can duplicate at a high frequency and cause insertion of the CTX element into the *V. cholerae* chromosome at a 17 base pair target site called attRS1. Sequences nearly identical to attRS1 (and apparently just as recombinationally active) exist at the ends of the RS1 sequences. These sequences are as follows:

attRS1 and flanking chromosomal sequences:

10 5'-TAAACCTAGAGACAAATGTTCTAGTGCGCATTATGTATGTTATGTTAAAT-3'
[SEQ.ID.NO:2]

Left side of RS1 and chromosomal junction:

5'-TAAACCTAGAGACAAATGTTCTAGTGCGCATTATGTGGCGCGGCAT...RS1...-3'
[SEQ.ID.NO:3]

15 Right side of RS1 and chromosomal junction:

5'-AAACCCTAGATTCCGCCGCCCTTAGTGCGCATTATGTATGTTATGTTAAAT-3'
[SEQ.ID.NO:4]

The attRS1 and a similar sequence present at the ends of RS1 are underlined. Note that the chromosomal sequence that flanks attRS1 is present on the left and the right side of RS1 with the only overlap being a 17 base pair sequence that is identical to attRS1 on the left end of RS1 and an 18 base pair sequence that matches 17/18 base pairs with attRS1.

25 Genetically engineered live attenuated cholera vaccines are theoretically safe only if they cannot revert or otherwise regain the capacity to produce cholera toxin. Strains which carry a single copy of the attRS1 sequence can efficiently acquire a new copy of the CTX element through DNA transfer by either P factor conjugation or bacteriophage transduction. Thus, deletions which render *V. cholerae* devoid of RS1 and attRS1 sequences can prevent a vaccine strain from reacquiring the CTX genetic element in nature through its own site specific recombination system. Such a deletion is present in strain Peru-2 and its derivatives.

- 11 -

Six mutant strains of *V. cholerae* with similar but not identical properties have been constructed. Four strains that carry the same two types of deletions (Type-1 and Type-2) as strains Peru-1 and Peru-2 were
5 constructed in *V. cholerae* strains isolated from patients in Bangladesh (P27459-Sm) and Bahrain (E7946-Sm). These four derivatives, Bang-1, Bang-2, Bah-1 and Bah-2 are also the subject of the invention because they vary in colonization and/or other properties (e.g., serotype) and
10 they are therefore potentially more suitable than the corresponding Peru strains for use as vaccines in other areas of the world.

Although the smaller Type-1 deletion present in the three strains Peru-1, Bang-1 and Bah-1 does not
15 remove all copies of RS1, this particular deletion affects the intestinal colonization properties of some of these strains more severely than the larger deletion present in Peru-2, Bang-2 and Bah-2.

Construction of Type-2 Deletion Mutations

20 A Type-2 deletion removes all sequences corresponding to the CTX genetic element including RS1 sequences and all copies of the attRS1 sequence (Fig.1). The Type-2 deletion was constructed by recombination between the chromosome of *V. cholerae* and the plasmid
25 sequences cloned on plasmid pAR62 as shown in Fig. 3. Plasmid pAR62 is a derivative of plasmid pGP60 and carries a Type-2 deletion wherein the HindIII fragment shown in Fig. 3 was deleted. Plasmid pGP60 was constructed by first generating a genomic library of
30 strain P27459 by inserting 20-30 kb Sau3A partially digested fragments into the BamHI site of plasmid pLAFR2 (Friedman et al., 1982, Gene 18:289). Colonies were screened by hybridization using probes derived from the ctx region (Mekalanos, 1983, Cell 35:253). A positive

- 12 -

colony was picked and the plasmid which was isolated therefrom was named pGP60. Restriction enzyme analysis of this plasmid confirmed that it contained all of the CTX element sequences and additional flanking DNA.

- 5 Plasmid pAR62 encodes resistance to tetracycline. This plasmid was introduced into a strain of *V.cholerae* by conjugation or electroporation followed by selection on media containing 3 µg/ml of tetracycline. Such a plasmid carrying strain was then screened by colony hybridization
- 10 with radioactive L-3 probe prepared as described in Goldberg and Mekalanos (J. Bacteriol. 165:723-731, 1986). Colonies carrying the Type-2 deletion inserted into the chromosome did not hybridize to the L-3 probe and surprisingly, occurred at a high frequency (i.e.,
- 15 about 1% of the colonies screened). Southern blot analysis was used to confirm the presence of the expected deletions in these strains.

Construction of Core (Type-1) Deletions

- A "core deletion" removes only sequences
- 20 corresponding to the core of the CTX element but leaves behind a copy of the RS1 element on the chromosome (Goldberg et al., J. Bacteriol. 165:723-731, 1986) (Fig. 2.). These deletions occur spontaneously through homologous recombination between RS1 sequences located on
- 25 the right side and left side of the core region as shown in Fig. 2. Colonies of *V. Cholerae* that contain core deletions can be identified in two ways. First, if the strain carries a selectable marker such as a gene encoding kanamycin resistance inserted in the core
- 30 region, then the core deletion renders such a strain sensitive to kanamycin (Goldberg et al., J. Bacteriol. 165:723-731, 1986). Second, colonies that contain the core deletion can also be identified by colony hybridization using radioactive CT-1 probe which does not
- 35 hybridize to strains carrying this deletion (Goldberg et

- 13 -

al., J. Bacteriol. 165:723-731, 1986). By either method, colonies that carry these deletions occurred at a frequency of about 1 per 1000 colonies screened.

Analysis by Southern blot hybridization was then used to
5 confirm the expected deletions in these strains.

An Assay for Functional attRS1 Sequences Based Upon
Integration of Plasmid pGP52

The plasmid pGP52 is a suicide plasmid which is only capable of replicating in strains of *E. coli* such as
10 SM10 λ pir (Pearson et al., 1990, Res. Microbiol. 141:893). Plasmid pGP52 was constructed by first digesting the plasmid pGP7 (Mekalanos, 1983, Cell 35:253) with ClaI and SphI. This plasmid contains two RS1 sequences (termed RS1 and RS2) derived from the *V. cholerae* strain E7946-
15 Sm. A fragment of DNA which contained the RS1 sequences was cloned into pBR322 and the resulting plasmid was named pGP20. This plasmid was then digested with EcoRV (which cuts within the RS1 sequences). When this plasmid was religated a new plasmid termed pGP20R was generated
20 containing a hybrid version of RS2 called RS2*, wherein the hybrid RS2 sequences were flanked by core sequences. An SspI-SphI fragment of RS2 was then subcloned into the suicide plasmid pJM703.1 which had been digested with NruI and SphI. The plasmid pJM703.1 is described in
25 Miller et al. (Proc. Natl. Acad. Sci. USA 81:3471). The resulting plasmid was called pGP52. A diagram depicting the construction of pGP52 is shown in Fig. 4.

When pGP52 is transferred by conjugation into *V. cholerae* strains which contain attRS1 sequences, it
30 integrates into the *V. cholerae* chromosome by means of a site-specific recombination event between the attRS1 sequence on the chromosome and the attRS1 sequence present on the plasmid. Integration events such as these can be quantitated by determining the number of colonies
35 that stably maintain (i.e., are non-selected) ampicillin

- 14 -

resistance because resistance to ampicillin is encoded by pGP52. Confirmation of integration can be obtained in Southern blot hybridization experiments. If the *V. cholerae* strain to be tested has functional attRS1 sequences then integration will be observed in the test. If the strain does not contain functional attRS1 sequences, integration will not occur.

In order to assess the ability of the various vaccine candidates to serve as recipients for pGP52, the following experiments were performed. Donor *E. coli* strain SM10 λ pir pGP52 was mixed with the recipient *V. cholerae* test vaccine strain in 5 ml of Luria broth at concentration of 10^7 cells from each strain per culture. The mixture was incubated at 37°C for 5 hours at which time it was diluted 1:100 into fresh Luria broth containing 100 μ g/ml of streptomycin. The purpose of the streptomycin is to select against the *E. coli* donor strain by killing it. Thus, only the streptomycin resistant *V. cholerae* recipient strains are capable of growth. This culture was incubated until the growth rate of the cells reached saturation. The cultures were diluted again and further incubated until each cell had replicated a total of 20 times in the absence of any positive selection for pGP52. This culture was then diluted and plated on two separate media compositions in order to quantitate the number of viable colonies. One of these media is Luria broth which does not contain any antibiotics. The number of colonies appearing on these plates represents the total number of cells in the culture. The other medium is Luria broth which contains ampicillin. The number of colonies appearing on these plates represents the number of integration events that occurred following conjugation. The results are expressed as a ratio of stable integration events/total

- 15 -

number of viable cells and are presented in Table 1 below.

Table 1. Representative Integration Data on Peru Vaccine Strains

<u>Strain</u>	<u>Stable Integration events/total # viable cells</u>
5 Peru-1	5.2×10^{-5}
Peru-2	Not detectable ($< 5 \times 10^{-8}$)
Peru-3	Not detectable ($< 5 \times 10^{-8}$)
Peru-4	Not detectable ($< 5 \times 10^{-8}$)
Peru-5	Not detectable ($< 5 \times 10^{-8}$)

10 Based on these data it is evident that strain Peru-1, which contains two copies of the attRS1 sequences is capable of integrating the plasmid pGP52 into its chromosome at a frequency that is at least 1000-fold higher than any of the other strains tested, all of which
15 lack any attRS1 sequences.

Serological Characterization of Vaccine Strains

 The vaccine strains Peru-2, Bang-2, and Bah-2 were characterized further in terms of their serological and colonization properties. The data presented in Table 2
20 demonstrate that each derivative retained its expected serotype (i.e., the serotype of each of the mutants respective parental strain) when freshly harvested bacterial cells were tested by slide agglutination using Difco *V. cholerae* 01 Inaba or Ogawa typing serum. This
25 result indicates that these strains still express LPS antigens. Other tests demonstrate that these mutant strains are motile, prototrophic, and still express Tcp pili. Thus, the mutants express a number of properties that are important for their ability to be useful as live
30 vaccine strains.

- 16 -

Colonization Properties of the Vaccine Strains and Core
Deletion Mutants

To test the colonization properties of these vaccine strains, a mouse intestinal competition assay was
5 used as described in Taylor et al. (Proc. Natl. Acad. Sci. USA. 84:2833-2837, 1987) which has been shown to correlate accurately with the colonization properties of mutant strains when they are subsequently tested in human
volunteers (Herrington et al., J. Exper. Med. 168:1487-
10 1492, 1988). The assay measures differences in colonization of a mutant strain by comparing its ability to compete for growth and survival with another closely related or isogenic strain. In this assay, the mutant and competing strains were mixed in a ratio of
15 approximately 1:1 and then approximately one million cells of this mixture were introduced to the stomach of 3-5 day old suckling CD-1 mice. After 24 hours, the mice were sacrificed, the intestine was dissected, homogenized, and plated on bacteriological media
20 containing streptomycin which selects for both strains. Colonies that grew after overnight incubation are then tested for additional markers which differentiate the mutant strain from the competing strain (i.e., resistance to kanamycin or hybridization with appropriate
25 radioactive DNA probes; see legend of Table 3).

As shown in Table 3, Bang-2, and Bah-2 both exhibited a mild intestinal colonization defect that resulted in approximately 4-13 fold greater recovery of the isogenic competing strains than the mutant strains
30 after 24 hours of growth in the mouse intestine. Also shown in Table 3, are results from competition assays involving core deletion mutant strains Peru-1, Bang-1 and Bah-1. Like the Type-2 deletion strains Bang-2 and Bah-2, these core deletion mutants were defective in
35 colonization relative to their isogenic competing

- 17 -

- strains. Because core deletions remove sequences corresponding to the core of the CTX element (Fig. 1 and 3), these data suggest that the core of CTX element encodes an "intestinal colonization factor, or ICF".
- 5 Cholera toxin by itself is not an ICF. Strains SM44 and SM115 which are defective in cholera toxin production due to a deletion in the *ctx* genes and insertion of a gene encoding kanamycin resistance as described in Goldberg and Mekalanos (J. Bacteriol. 165:723-731, 1986)
- 10 outcompete their respective mutant strains (Bang-1, Bang-2 and Bah-1, Bah-2) in the intestinal competition assay. Thus, it is apparent that SM44 and SM115 make ICF even though they do not produce cholera toxin, while the mutants do not. Furthermore, because the CTX core region
- 15 was the only DNA that is deleted in both core as well as Type-2 deletions and mutants carrying both types of deletions were similarly defective in colonization, it can also be concluded that ICF is encoded by the core region of the CTX element as shown in Fig. 1.
- 20 Recently, a new toxin called ZOT has been found to be encoded by the core region (Baudry et al., 1992, Infect. Immun. 60:428-434). We have evidence that mutations in the *ZOT* gene do not produce the colonization defect observed in Type-1 or Type-2 deletion mutants.
- 25 Accordingly, ICF is designated as a separate and distinct property from ZOT. The vaccine strains described herein carrying Type-1 or Type-2 deletions are defective in ICF.

In contrast, strain Peru-2 exhibited no significant defect in intestinal colonization relative to

30 its competing strain C6709-Sm (Table 2). However, the total cell yield of either strain C6709-Sm or Peru-2 in the mice was typically 10-100 fold less than strains SM44 or SM115, suggesting that the Peru strain C6709-Sm and its derivative Peru-2 may already carry an undefined

- 18 -

colonization defect. Since deletion of the core of all or part of the CTX element did not cause a further defect in the colonization of either strain Peru-1 or Peru-2, it can be concluded that strain C6709-Sm is partially
 5 defective in ICF already even though it carries DNA sequences that correspond to the CTX core region. Deletion of the entire CTX region as defined by the Type-2 mutations present in strains Peru-2, Bang-2 and Bah-2 assures that the genes for ICF cannot reactivate and
 10 become functional in the vaccine derivatives. The Type-2 deletion of ICF genes apparently causes a mild colonization defect. Such may be useful as an attenuating mutation in cholera vaccine development, because wild type ICF may be responsible for undesirable
 15 levels of toxicity.

Table 2. Properties of Mutant Strains

<u>Mutant Strains</u>	<u>Parental Strain</u> *	<u>Serotype</u>	<u>Type of Deletion</u>
Peru-2	C6709-Sm	Inaba	Type-2
Bang-2	P27459-Sm	Ogawa	Type-2
20 Bah-2	E7946-Sm	Inaba	Type-2

* Note that the designation "Sm" behind the strain name refers to streptomycin resistance. This is a spontaneously selected strain which is resistant to 100 μ g/ml of streptomycin and was the result of a spontaneous
 25 point mutation in the gene for a ribosomal protein. This resistance marker is not associated with a plasmid or transposon and is therefore not transmissible to enteric flora. Because all mutant strains are derived from the indicated parental strains, all mutant strains are also
 30 resistant to streptomycin.

- 19 -

Table 3. Infant Mouse Colonization Competition Assays^a

Mutant Strain	Competing Strain	Input Ratio	Output Ratio
		Mutant/Competing Strain	Mutant/Competing Strain
Bang-2	SM44 ^b	0.61	0.16
5 Bah-2	SM115 ^c	0.92	0.07
Peru-2	C6709-Sm ^d	0.74	0.65
Bang-1	SM44 ^b	0.85	0.05
Bah-1	SM115 ^c	0.61	0.04
Peru-1	C6709-Sm ^d	0.89	0.94

- 10 ^a Infant mouse colonization assays were performed according to the method described in Taylor et al. (Proc. Natl. Acad. Sci. USA. 84:2833-2837, 1987). The ratio of strains was determined by either differential sensitivity to antibiotics or by colony hybridization with
- 15 appropriate probes as described in the additional footnotes below.

^bStrain SM44 has been described in Goldberg and Mekalanos (J. Bacteriol. 165:723-731, 1986) and is a kanamycin resistant derivative of the parental strain P27459-Sm.

- 20 The gene encoding kanamycin resistance in SM44 was inserted in the *ctx* locus. Because Bang-1 and Bang-2 were derivatives of P27459-Sm competition with SM44 measures colonization differences that can be attributed to the effect of the Type 2 rather loss of *ctx*. Strains
- 25 Bang-1 and Bang-2 were sensitive to kanamycin and were differentiated from SM44 in these competitions assays by scoring colonies for resistance to 30 µg/ml kanamycin.

- ^cStrain SM115 has been described in Goldberg and Mekalanos (J. Bacteriol. 165:723-731, 1986) and is the
- 30 kanamycin resistant derivative of the parental strain E7946-Sm. The gene encoding kanamycin resistance in SM115 was inserted in the *ctx* locus. Because Bah-1 and Bah-2 are derivatives of P27459-Sm competition with SM115 measures colonization differences that can be attributed

- 20 -

to the effect of the Type 2 deletion rather than loss of
ctx. Strains Bah-1 and Bah-2 were sensitive to
kanamycin and were differentiated from SM115 in these
competitions assays by scoring colonies for resistance to
5 30 µg/ml kanamycin.

^dStrain C6709-Sm is the parental strain of Peru-1 and
Peru-2. Peru-2 carries a Type -2 deletion while Peru-1
carries a core deletion. Both these deletions remove the
ctx genes and thus both Peru-1 and Peru-2 were negative
10 in colony hybridization blots when probed with the CT-1
probe described in Goldberg and Mekalanos (J. Bacteriol.
165:723-731, 1986) while strain C6709-Sm was positive
using the same probe. Thus, both Peru-1 and Peru-2 were
differentiated from C6709-Sm in these competitions assays
15 by scoring colonies for hybridization with the CT-1
probe.

The mutant strains described can be further
improved as vaccine candidates by creating additional
mutations within each strain that will serve to enhance
20 the safety and immunogenicity of the vaccine.

With regard to safety, a second mutation can be
introduced into the *recA* gene of any of the strains
described above, which mutation is designed to inactivate
that *recA* gene. Such double mutant strains will
25 therefore be defective in recombination and will be
unable to recombine with wild type strains of *V. cholerae*
in the environment. Thus, they will be incapable of
acquiring wild type toxin genes and expressing the CTX
element. Immunogenicity can also be improved by
30 introducing additional mutations into each strain which
will allow that strain to express cholera toxin related
antigens (e.g., the B subunit of cholera toxin) or other
heterologous antigens, e.g., the nontoxic B subunit of
Shiga-like toxin or various CFA antigens of

- 21 -

enterotoxigenic *E. coli* strains, Shiga-toxin, anthrax toxin, *Pseudomonas* endotoxin A, pertussis toxin, tetanus toxin; antigens from Herpes virus, rubella virus, influenza virus, mumps virus, measles virus, poliovirus, poliomyelitis virus, antigenic fragments from the HIV capsid; and immunogenic polypeptides from eukaryotic parasites causing malaria, pneumocystis pneumonia, and toxoplasmosis (Karjalainen et al., 1989, Infect. Immun. 57:1126; Perez-Casal et al., 1990, Infect. Immun. 58:3594). Thus, a series of mutated derivatives can also be useful in the invention, each incorporating additional properties that render the strains safer, genetically more stable and more broadly immunogenic. The construction of such derivatives is described below.

15 Construction of *recA/ctxB* Alleles

Cholera toxin B subunit is known to be a nontoxic, highly immunogenic molecule that is capable of inducing cholera toxin neutralizing antibodies. In order to generate more immunogenic vaccine strains, a new copy of the *ctxB* gene was introduced into the vaccine strains containing the Type-2 deletions described above (because Type-2 deletions remove all of the coding sequence for the cholera toxin B subunit). This was accomplished in a series of steps that are described below.

25 First, a promoterless copy of the *ctxB* gene was constructed using the polymerase chain reaction (PCR). For PCR, the downstream primer was designed so that the *ctxB* coding sequence could be synthesized in such a way as to eliminate the *attRS1* site that lies just downstream from the stop codon in the *ctxB* gene. This primer had the following sequence: 5'-

GGGCTAAAGTTAAAAGACAAATATTTTCAGGC-3' [SEQ.ID.NO:5]. The upstream primer was designed so that only the last 24 carboxyterminal amino acid residues of the A2 subunit

- 22 -

could be encoded by the product of the reaction. This primer had the following sequence:

5'-GGGTAGAAGTGAAACGGGGTTTACCG-3' [SEQ.ID.NO:6].

All other nucleotides in the DNA encoding the A subunit
5 were excluded from the reaction. The DNA encoding the
carboxyterminal amino acids of CtxA2 were retained in the
final product to allow for translational coupling of *ctxB*
gene expression. Since the toxic activity associated
with cholera toxin is derived from the CtxA1 polypeptide,
10 all sequences encoding the A1 polypeptide were excluded
from the PCR reaction.

PCR was performed using the *ctxB* primers as
described above using *V. cholerae* DNA from the Peruvian
strain, C6709-Sm (Fig. 5). The product of the reaction,
15 a 0.6 kilobase pair fragment, was cloned into plasmid
PCR100. This fragment was then cut out of the plasmid as
a 0.6 kilobase pair *SpeI*-*EcoRI* fragment and was cloned
into two individual acceptor plasmids, *XbaI*-*EcoRI*
digested pRT41 and *XbaI*-*EcoRI* digested pVC100. The
20 resulting plasmids, pJM411 and pJM1001, then each encode
a copy of the *ctxB* gene under the control of either the
ctx promoter (*ctxP*) or the *htpG* promoter (*hptP*) of *V.*
cholerae, respectively. These plasmids were then
transferred to the nontoxigenic strain *V. cholerae* 0395-
25 NT (Mekalanos et al., 1983, Nature 306:551 and U.S.
Patent No. 4,935,364), generating two new strains termed
0395-NT pJM411 and 0395-NT pJM1001. The amount of
cholera B subunit produced by each strain was measured by
GMI ELISA. Strain 0395-NT pJM411 produced 30 µg/ml,
30 while strain 0395-NT pJM1001 produced 100 µg/ml in LB
culture supernatant fluids. These results demonstrate
that the PCR product was a functional *ctxB* gene encoding
an antigenic cholera B subunit capable of binding to
ganglioside GMI and was therefore similar to that
35 secreted by normal wild type *V. cholerae*.

- 23 -

In the next step, EcoRI-BamHI fragments of DNA specifying the promoter-ctxB constructs were subcloned into the suicide recA plasmid pGP84. This plasmid contains a *V. cholerae* chromosomal DNA insert that
5 corresponds to the DNA which flanks the *recA* gene of *V. cholerae* (i.e., an internal deletion of *recA*). Plasmid pGP84 is a derivative of suicide plasmid pJM703.1 (Miller et al., 1988, J. Bacteriol. 170:2575) and encodes sequences corresponding to the flanking regions of the
10 *recA* gene of *V. cholerae* (Goldberg et al., 1986, J. Bacteriol. 165:715) including a BglII-PvuII fragment on the left side and an XbaI-EcoRI fragment on the right side. A 1.3 kb fragment encoding kanamycin resistance is positioned between these two fragments. Plasmid pGP84
15 also contains a NruI-BamHI fragment encoding sensitivity to streptomycin. This latter fragment is derived from plasmid pN01523 (Dean, 1981, Gene 15:99). When pGP84 is digested with XbaI, the 1.3 kb fragment is removed and other XbaI fragments can be inserted into this deleted
20 *recA* region.

The subcloning was accomplished as follows: Each of the two EcoRI-BamHI fragments specifying the promoter-ctxB constructs were modified by the addition of XbaI linkers. They were individually ligated to XbaI digested pGP84 to
25 generate two new plasmids pJM84.1 and pJM84.2, each of which contains DNA specifying the *htpP-ctxB* and the *ctxP-ctxB* constructs respectively (Fig. 6).

Next, plasmids pJM84.1 and pJM84.2 were transferred into *V. cholerae* strains Peru-2, Bang-2 and
30 Bah-2 and ampicillin resistant colonies were selected. Because these plasmids are incapable of replication in *V. cholerae*, they integrate into the host cell chromosome by homologous recombination generating the structure shown in Fig. 6. Both plasmids also encode a gene for
35 streptomycin sensitivity which allows for positive

- 24 -

selection against a plasmid integration event in strains that are streptomycin resistant (i.e., strains Peru-2, Bang-2 and Bah-2). Thus, when strains that have a plasmid integrated into the chromosomal DNA are grown on medium containing 2 mg/ml streptomycin, colonies that have reverted to ampicillin sensitivity can be isolated. Strains that had now crossed out the integrated plasmid in such a way as to leave behind the *recA* deletion mutation together with the *ctxB* construct were then selected from among these latter strains. These strains were easily identified as having the following properties:

1. They were ampicillin sensitive.
2. They were killed in the presence of 0.1 ml methyl methane sulfonate per ml of LB, a characteristic phenotype of *recA*⁻ cells.
3. They produced the cholera B subunit as measured by GMI-ELISA.
4. Southern blot analysis using *recA* and *ctxB* probes confirmed that they contained DNA fragments consistent with the presence of the *ctxB* construct and deletion of the appropriate *recA* sequences.

Bacterial strains that were isolated following the procedure described above are as follows:

25	STRAIN	GENOTYPE
	Peru-3	<i>attRS1</i> deletion, <i>recA</i> :: <i>htpP-ctxB</i> , <i>str</i>
	Peru-4	<i>attRS1</i> deletion, <i>recA</i> :: <i>ctxP-ctxB</i> , <i>str</i>
	Bang-3	<i>attRS1</i> deletion, <i>recA</i> :: <i>htpP-ctxB</i> , <i>str</i>
	Bah-3	<i>attRS1</i> deletion, <i>recA</i> :: <i>htpP-ctxB</i> , <i>str</i>
30	Bah-4	<i>attRS1</i> deletion, <i>recA</i> :: <i>ctxP-ctxB</i> , <i>str</i>

Construction of *lacZ-ctxB* Alleles

The *recA* mutation contained within the vaccine strains described above renders the strains deficient in homologous recombination. In order to produce candidate vaccines that were still capable of homologous recombination, the *ctxB* gene was inserted into the *lacZ* gene of *V. cholerae* as described below.

- 25 -

The plasmid pCG698 which encodes the *lacZ* gene of *V. cholerae*, contains a unique *HpaI* site in the middle of the *lacZ* coding sequence. The plasmid pCG698 was constructed as follows: The β -galactosidase gene of *V. cholerae* was cloned from a library of chromosomal DNA fragments from strain E7946 as described (Mekalanos, 1983, Cell 35:253). It was found to express β -galactosidase and following restriction enzyme mapping, was found to contain a 6 kb insert containing 2 *HpaI* sites in the *lacZ* gene each of which was separated by 2.1 kb of DNA. This plasmid was linearized with *HpaI* and *XbaI* linkers were ligated to the ends. An *EcoRI*-*BamHI* fragment containing the *ctxP*-*ctxB* construct was removed from pJM411 as described above, the ends were modified by the addition of *XbaI* linkers and the fragment was ligated into the similarly modified pCG698. The resulting plasmid pJM6891, now contained the *ctxP*-*ctxB* construct inserted into the middle of the *lacZ* gene. This plasmid was transferred into *V. cholerae* strains Peru-2, Bang-2 and Bah-2 and each resulting strain was screened for growth in the presence of X-gal. White colonies containing an inactivated *lacZ* gene were picked and purified. Strains that contained an integrated copy of the *lacZ::ctxP-ctxB* sequences into the host cell chromosome were obtained by curing the bacteria of pJM6891 by growth in the absence of ampicillin. The presence of the appropriate sequences was confirmed by Southern blot analysis and the ability of these bacteria to produce cholera toxin B subunit was confirmed by GMI-ELISA. Bacterial strains isolated following this procedure are as follows:

STRAIN	GENOTYPE
Peru-5	<i>attRS1</i> deletion, <i>lacZ::ctxP-ctxB</i> , <i>str</i>
Bang-5	<i>attRS1</i> deletion, <i>lacZ::ctxP-ctxB</i> , <i>str</i>
Bah-5	<i>attRS1</i> deletion, <i>lacZ::ctxP-ctxB</i> , <i>str</i>

In order to characterize some of these carrier cholera vaccine candidates with regard to mouse

- 26 -

colonization, mice were infected with the strains listed below. The strain TCP2, a derivative of 0395-N1 which contains a *TcpA* deletion and does not colonize the intestine of human volunteers, served as a control. Five mice were used for each strain. At 24 hours post-infection, the upper intestine was removed from each mouse, homogenized and assayed for the number of *V. cholerae* present using a simple plating assay. The results are presented in the table below. Essentially, no TCP2 bacteria were detected in the intestines of mice infected with TCP2 and thus the values given below represent the number of bacteria of each strain that colonized the mouse intestine above a background level of zero.

15	Strain	CFU per mouse ^a	Genotype/Construct ^b
	Peru-3	9.4×10^5	<u>attRS1</u> deletion #2, <u>recA::htpG-ctxB</u>
	Peru-2	2.5×10^6	<u>attRS1</u> deletion #2, <u>recA::ctx-ctxB</u>
	Peru-4	6.0×10^6	<u>attRS1</u> deletion #2, <u>lacZ::ctx-ctxB</u>
	Peru-5	6.6×10^6	<u>attRS1</u> deletion #2, <u>recA::htpG-ctxB</u>
20	Bang-2	9.9×10^6	<u>attRS1</u> deletion #2, <u>recA::htpG-ctxB</u>
	Bang-3	2.7×10^7	<u>attRS1</u> deletion #2, <u>recA::htpG-ctxB</u>

a Colony forming units recovered per mouse (average of five mice).

b The construct attRS1 deletion #2 is a Type 2 deletion constructed with plasmid PAR62, described in Figure 3.

25 The construct recA::htpG-ctxB is a deletion of the recA gene and insertion of the cholera toxin B subunit gene under control of the heat shock promoter derived from the htpG of V. cholerae.

30 The construct recA::htpG-ctxB is a deletion of the recA gene and insertion of the cholera toxin B subunit gene under control of the cholera toxin promoter derived from the ctx gene of a hypotoxigenic strain 569B of V. cholerae.

The construct lacZ::ctx-ctxB is an insertion in the lacZ gene of V. cholerae that is composed of the cholera toxin B subunit gene under control of the cholera toxin promoter derived from the ctx gene of a hypotoxigenic strain 569B of V. cholerae.

The results suggest that the presence of the recA::htpG-ctxB allele serves to reduce the ability of the Peru-derived strains to colonize the intestine (compare, for example, Peru-3 with Peru-2). However, the effect of this construct on colonization of the Bang-derived strain was less marked (compare Bang-3 with Bang-

- 27 -

- 2). In general, introduction of the constructs wherein *ctxB* is under the control of its own promoter had less effect on colonalization than the constructs wherein it was placed under the control of the heat shock promoter.
- 5 It should be noted that strains Peru-2, Peru-3 and Bang-3 vary in their colonalization properties over a 28-fold range. It is well within the art following the protocols described above, to isolate additional vaccine candidates that vary even more widely in their colonalization
- 10 properties.

In summary, the data demonstrate the feasibility of using genetic engineering techniques to generate novel *ctxB*-containing *V. cholerae* strains wherein the expression of the *ctxB* gene is placed under the control

15 of either of two *V. cholerae* promoters (*ctxP* and *htpP*). The engineered genes can be recombined into the *V. cholerae* chromosome into target genes such as *recA* or *lacZ* to generate strains which stably express large amounts of cholera toxin B subunit (for example, strains

20 Peru-3, Peru-4 and Peru-5).

Isolation of spontaneous soft agar penetration-defective strains of *V. cholerae*

Mutants of *V. cholerae* which are defective in soft agar penetration can be useful in the production of

25 vaccines. The rationale for utilizing these mutants is as follows. The mucous layer of the intestine is thought to be viscous and mutants defective in penetration of soft agar might be deficient in penetration of this mucous. Although defective in penetration through mucous, these

30 mutants may still present antigen to the Peyer patches which are not covered by a thick mucous gel and which include antigen-sampling cells specific for IgA antibody production. As a result, penetration defective mutants are predicted to have low reactogenicity, yet be highly

- 28 -

antigenic, and these characteristics are desirable for a live vaccine. Although non-motile mutants are one class of mutants defective in penetration of soft agar, other types of mutations may also result in a soft agar

5 penetration-defective phenotype (i.e., a swarming phenotype) and may be useful for vaccines. In keeping with this line of reasoning, completely non-motile mutants, i.e., mutants unable to swarm in agar-free media, may be useful candidate vaccines.

10 To obtain such mutants, soft agar can be used to assess the ability of bacteria to penetrate a media of high viscosity (soft agar media which is 0.25 - 0.4% agar), as described below. One such soft agar penetration-defective vaccine with a high therapeutic
15 value is Peru-14.

Peru-14 is soft agar penetration-defective, and, in addition, over 50% of Peru-14 cells are filamentous, with a spiral-like appearance and having a cell length of greater than 5 normal cell lengths (25nM, as opposed to
20 the wild-type cells length of 5nM).

Peru-14 was isolated as a soft agar penetration-defective derivative of the triply-deleted Peru strain (Peru-3) ($ctxA^-$, att^- , and $recA^-$) that was free from side effects but still retained the ability to colonize
25 vaccinees as shown in below (Table 7).

Although Peru-14 was isolated based upon the theory stated above, this theory of function may or may not accurately and completely explain the effectiveness of Peru-14 as a vaccine. The usefulness of Peru-14 as an
30 effective vaccine does not depend on the correctness of this theory.

- 29 -

Table 7Outcome of Immunization with Freshly Harvested Peru-14
Cholera Vaccine

of Dose (cfu) Day	Volunteer #	Symptoms	Stool	Duration	
				Excretion (days)/Peak	
2x10 ⁶ 3/3	28	Gas	Formed		
15/2	29	Cramps	Formed		
-	30	None	Formed		--
4/4	33	None	Formed		
20 4/1	34	None	336g*		
3/3	35	None	Formed		
9x10 ⁸ 25/1	25	None	Formed		
3/1	26	Gas	Formed		
2/2	27	Headache	Formed		
30 7/4	31	Nausea, Loss of Appetite	Formed		
5/3	32	None	Formed		
35/1	36	Cramps	63g+		

- * Volunteer had painless semi-solid stool at 72 hours post-immunization. Stool was culture-negative for Peru-14.
- 40 + Volunteer had two small liquid stools at 48 hours post-immunization. Stools were culture-positive for Peru-14.

Specifically, the Peru-14 soft agar penetration-defective strain was produced as follows. Peru-3 was grown overnight in LB broth containing 100 µg streptomycin sulfate at 30°C. The culture was diluted to approximately 2000 cfu/ml and 0.1 ml was plated onto LB plates containing 100 µg streptomycin. After incubating the plates overnight at 30°C, approximately 1000 colonies

- 30 -

were toothpicked into soft agar plates (LB broth + 0.45% Bacto-agar) and incubated overnight at 30°C. The inoculating toothpick is inserted only 1-2 mm into the surface of the soft agar plate. Of the 1000 colonies
5 picked, 25 appeared to be non-penetrating. Non-penetrating isolates appear as colonies of approximately 2 mm in diameter, whereas penetrating isolates swarm on and within agar the agar to a diameter greater than 5 mm. These colonies were repicked into soft agar once again,
10 along with a known non-penetrating, non-motile cholera strain and the original Peru-3 strain. One colony of the 25 was non-soft agar penetrating (when compared to the controls). This colony, designated Peru-14, was still Inaba positive with agglutination sera, and produced the
15 same level of B-subunit toxin as Peru-3 when tested in the B-subunit ELISA. The methods described above can be used for isolating soft agar penetration defective mutants of any *V. cholerae* strain. Non-revertable penetration-defective mutants, such as those harboring a
20 genetic deletion, can be made using the methods described above.

Bengal strains

A highly unusual non-01 virulent strain has recently been discovered to be responsible for a cholera
25 epidemic on the Indian sub-continent. Survivors of earlier 01 serogroup epidemics are not immunologically protected against this strain.

This strain has been deposited with the American Type Culture Collection (ATCC) in Rockville, MD. Bengal
30 can be attenuated as described above for the other strains, e.g., by one or more of the following mutations: *ctx*⁻, *att*⁻, or *recA*⁻, or a soft agar-defective phenotype, Bengal-2 ("Beng-2") and Bengal-3 ("Beng-3"), are genetically equivalent to Peru-2 and Peru-3. Such an
35 attenuated Bengal strain may be combined with one of the

- 31 -

above-described attenuated Peru strains to provide a dual or multi-cholera strain vaccine.

Human testing of Peru-3 and Peru-5

Human studies of the efficacy of Peru-3 and Peru-5 were performed as follows.

Blood samples were drawn from volunteers prior to immunization and at 7, 14, 21, and 28 days post immunization. The *V. cholerae* antibodies in their blood stream were measured and levels are shown in Table 4.

10 The immunogenicity of vaccine prototypes Peru-3 and Peru-5 were evaluated in human volunteer studies. Volunteers ingested freshly harvested Peru-3 or Peru-5 at 3 different doses in 100ml of 10% sodium bicarbonate. Peru-3 and Peru-5 were also shown to induce antitoxin

15 antibodies (Table 5). In addition, Peru-3 and Peru-5 were shown to protect volunteers from challenge with a wild-type El Tor *V. cholerae* (strain N16961, Table 6). We conclude that Peru-3 in particular provokes a potent immune response (Tables 4 and 5) and confers protection

20 from cholera in human studies (Table 6).

- 32 -

Table 4

Vibriocidal Titers After Immunization with Peru-3 or Peru-5 (July 1992)

5	Strain(cfu)	Volunteer	Pre	7	14	21	28	Peak
	Peru-3 (4×10^6)	1	50	1600	6400	6400	6400	6400
		2	<100	50	100	50	100	100
		5	<100	1600	6400	400	400	6400
10	Peru-3 (1×10^8)	7	<100	400	1600	400	400	1600
		12	<100	400	800	400	200	800
		13	<100	3200	6400	3200	1600	6400
15	Peru-5 (2×10^6)	11	<100	1600	6400	3200	6400	6400
		14	<100	200	6400	3200	1600	6400
		15	<100	800	6400	1600	3200	6400

Heat activated serum samples were serially diluted into microtiter wells, mixed with log phase *V. cholerae* (final concentration of 5×10^7) and guinea pig complement (final concentration of 11%) and incubated at 37°C for 1 hour.

- 20 Brain-Heart-Infusion broth was then added to plates and incubated at 37°C for 2.75 hours. Values in table represent the reciprocal titers at which antibody-mediated killing of *V. cholerae* was 50% or greater.

Table 5

25 Cholera Antitoxin Titers after immunization with Peru-3 or Peru-5 (July 1992)

30	Strain(cfu)	Volunteer	.2	7	14	21	28	Peak Increase (fold)
	Peru-3(4×10^6)	1	8	8	32	32	32	4
		2	<2	<2	<2	<2	<2	None
		5	2	64	64	2	256	14
35	Peru-3(4×10^8)	7	2	2	4	4	4	2
		12	<2	2	4	4	4	4
		13	<2	<2	<2	<2	<2	None
40	Peru-5(2×10^6)	11	4	4	4	4	4	None
		14	2	2	2	2	2	None
		15	8	8	8	8	8	None

- 45 Serum samples were serially diluted into pre-treated, ganglioside/cholera toxin B-subunit coated 96 well microtiter plates and incubated at 37°C for 30 minutes. Following 3 washes with PBS, goat anti-human antibody-alkaline phosphatase conjugate (1/1000) was added and incubated at 37°C for 30 minutes. Following 3 washes with PBS, 2mg/ml PNPP was added to each well and incubated for 15 minutes. Reaction was stopped with 0.1M K_2PO_4 and read at an O.D. of 405nm. Values on the table represent the reciprocal titers and the increase of day-2 compared to peak titer.

- 33 -

Table 6

Outcome for Volunteers Challenged with 2×10^6 cfu of Vibrio cholerae (N16961) wild-type Organisms (November 1992)

5	Subject Number	Previous Vaccination	Initial Dose	Symptoms	Diarrhea (grams)	Onset of Symptoms
	1	Peru-3	6 logs	None	Formed	
10	2	Peru-3*	6 logs	Tired, gurgling	534	18-48 hours
	5	Peru-3	6 logs	None	3	
	7	Peru-3	8 logs	None	23	36 hours
	11	Peru-5	6 logs	None	Formed	
	12	Peru-3	8 logs	None	Formed	
15	14	Peru-5	6 logs	None	Formed	
	15	Peru-5	6 logs	None	Formed	
	22	Control		T 100.7 F, HA, nausea LOA, gurgling, cramps	1443	24 hours
20	23	Control		None	769	24 hours
	24	Control		T 99.6 F, HA, malaise, gurgling, cramps	904+	40 hours
25						

- Did not colonize or subsequently seroconvert after vaccination
- + Two liquid stools not weighed due to urgency

Construction of *V. cholerae* vaccines expressing

30 heterologous antigens

The procedures described above can be applied by any artisan skilled in the art for the construction of derivatives of Peru-2, Bang-2, Bah-2, Peru-14, and related strains which are capable of expressing a wide
 35 variety of foreign or heterologous antigens, e.g., antigens that are not normally expressed in *V. cholerae*. Such derivatives, when used as live vaccines, would be expected to induce a strong immune response against both *V. cholerae* antigens and the foreign antigen that it
 40 encodes. Both systemic and local immune responses will likely be induced because vaccination with other prototype *V. cholerae* vaccines has resulted in the induction of circulating IgG and local IgA antibodies

- 34 -

that are specific for both whole cell antigens (e.g., LPS) and as well as individual proteins such as cholera toxin B subunit (Herrington et al., 1988, J. Exp. Med. 168:1487-1492). A foreign antigen expressed by V.

5 *cholerae* would be expected to elicit an immune response similar to that of the individual cholera proteins.

The methods useful for the introduction of heterologous antigens into *V. cholerae* are similar to those described above for the re-introduction of the *ctxB*
10 gene into vaccine strains Peru-3, Peru-4, Peru-14, Peru-5, Bang-3, Bah-3 and Bah-4. Virtually any heterologous antigen can be inserted into *V. cholerae* using these methods.

The same protocol used to construct *ctxB*
15 containing strains under a novel promoter can be used to construct derivatives of Peru-2, Bang-2 and Bah-2 which are capable of expression virtually any heterologous antigen or antigens normally encoded by either bacteria, viruses, or parasites. The methods described in the
20 invention therefore teach generation of a multivalent *V. cholerae* vaccine "carrier strain" which can be manipulated to encode and express other antigens and can be administered to humans in order to immunize them against not only cholera, but other pathogens as well.

25 *V. cholerae*/enterotoxigenic *E. coli* vaccines

Vibrio cholerae vaccines which elicit antibodies against cholera toxin (CT) have been demonstrated to confer cross protection to human vaccinees against strains of heat-labile toxin (LT) producing
30 enterotoxigenic *E. coli* (ETEC) (Svennerholm, J. Infect. Dis., 149:884-893, 1984). Vaccinees were still vulnerable however to heat-stable toxin (ST) producing strains of ETEC. An attenuated strain of *Vibrio cholerae*, Peru-3, can be used as a vaccine vector harboring ETEC-derived
35 foreign genes encoding the major subunit of colonization

- 35 -

factor antigen CFA/IV fimbriae, and a genetic toxoid of ST. Such a vaccine vector will elicit i) anti-fimbrial antibodies, precluding binding of pathogenic ETEC strains to the human gut epithelium, and ii) anti-ST antibodies;
5 negating the diarrheal effects of ST. The result is a single dose orally administered live attenuated *V. cholerae* vectored ETEC vaccine.

The attenuated *V. cholerae* vectored ETEC vaccine may have one or more of the following advantages: i) it can
10 be lyophilized for long-term storage, ii) it requires no cold-chain, iii) it is orally administered, iv) it requires only a single-dose, v) it is cost effective, and vi) it protects against most ETEC strains.

A single-dose live oral vaccine directed against the
15 enteric pathogens, *V. cholerae* and enterotoxigenic *E. coli* (ETEC) is made by genetically engineering sequences encoding antigens from *E. coli* into the *V. cholerae* vaccine strains. In the construction of such vaccine strains, it is desirable to neutralize both colonization
20 and toxin production. This can be achieved by modifying an attenuated strain of *V. cholerae*, Peru-3 as described above.

Peru-3 strain already expresses cholera toxin B subunit which is nearly identical to the ETEC heat-labile
25 toxin B subunit, and elicits cross protective antibodies. The strain can be modified to express fimbrial antigens of ETEC and a chimeric protein made up of the oligomerization domain of cholera toxin A subunit and a mutant form of the ETEC heat-stable toxin. In this way,
30 the induction of immunity to both *V. cholerae* and *E. coli* can be accomplished.

The generation of the *V. cholerae*/ETEC vaccine strain is accomplished by utilizing common techniques in microbiology and molecular biology. The ability of the
35 strain to colonize animals and induce an immune response

- 36 -

can be analyzed in an established model of enteric infection of rabbits.

Cloning and expression of fimbrial antigens

The ability of ETEC to colonize the intestinal
5 epithelium of humans is mediated by serologically
distinct fimbriae known as colonization factor antigens
(CFAs) and putative colonization factors (PCFs). The
CFA/4 fimbriae is the principal colonization factor
identified in approximately one quarter to one third of
10 all ETEC clinical isolates. The gene encoding the major
subunit of a prototype member of the group (CS6) has been
cloned and sequenced by others.

The cloned CS6 gene carried on a high-copy number
plasmid was introduced to Peru-3 via
15 electrotransformation and maintained by culture in Luria-
Bertani broth containing 50 µg/ml of ampicillin. Whole
cell lysates of Peru-3 containing the CS6 sequences were
analyzed for protein antigen expression by denaturing
polyacrylamide gel electrophoresis and immunoblotting
20 using anti-CS6 polyclonal rabbit serum. Immunoblots were
developed using anti-rabbit IgG-alkaline phosphatase
conjugate and BCIP. Expression of the CS6 gene was
detected as production of a 17-kiloDalton protein. Thus
CS6 antigen can be expressed in Peru-3 for the
25 formulation of a vaccine.

In order to generate the candidate vaccine strain,
however, it is desirable to have the CS6 gene stably
maintained in the absence of antibiotic selection and to
have it expressed from a promoter that is actively
30 transcribed by *V. cholerae*. To that end, the polymerase
chain reaction (PCR) can be used to specifically amplify
the CS6 gene carried on a plasmid and to create unique
restriction endonuclease sites at its termini for
subsequent cloning into an ampicillin resistant,
35 streptomycin sensitive "suicide" vector which allows

- 37 -

integration onto the chromosome of *V. cholerae*. Specifically, PCR generated CS6 DNA flanked with a 5' PacI site and a 3' NotI site can be ligated with pJM6891 DNA which has been digested with PacI and NotI, placing
5 the CS6 gene under the control of the cholera toxin promoter. The ligation mixture can be introduced by electrotransformation into *E. coli* strain SM10pir which provides a specific trans-acting protein, known as pi, required by pJM6891 for replication. However, when
10 pJM6891 and its derivatives are introduced into *V. cholerae* (which lacks the pi protein), selection for resistance to 50 µg/ml of ampicillin requires that the plasmid integrate onto the chromosome. The site of integration is determined by the presence of *V. cholerae*
15 *lacZ* DNA sequences flanking CS6 which are identical to sequences on the *V. cholerae* chromosome and allow homologous recombination to occur. The resulting progeny is ampicillin resistant and harbors an integrated copy of the plasmid and CS6 sequences surrounded by repeated DNA
20 sequences of the *lacZ* gene.

The repeats can be resolved to remove the vector sequences (including the ampicillin resistance determinant), leaving the CS6 gene under control of the toxin promoter. This is performed by culturing the
25 strain in the presence of 2 mg/ml of streptomycin, selecting for the streptomycin resistance allele native to Peru-3 and against the streptomycin sensitivity allele introduced by the plasmid. After growth overnight in the presence of streptomycin, the culture is plated
30 for single colonies on LB agar containing 100 µg/ml streptomycin, and scored for sensitivity to 50 µg/ml ampicillin. Isolates that are streptomycin sensitive and ampicillin resistant will be analyzed by Southern blots of chromosomal DNA to determine if the expected
35 integration and excision events have occurred.

- 38 -

These isolates can be analyzed for level of antigen production using immunoblotting techniques. Production of CS6 fimbrial antigen can be evaluated under a variety of growth conditions known to affect transcription of the cholera toxin promoter. The effect of media pH (6.5 versus 8.0), temperature (30°C versus 37°C), NaCl concentration (50 to 500 mM) and amino acid concentration (0 to 25 mM) on the level of CS6 expression can be determined.

10 The candidate vaccine strain Peru-3/CS6 can then be used in a rabbit model to demonstrate safety and immunogenicity. Since the human clinical isolates of ETEC that produce CFA antigens are typically not pathogenic to laboratory animals, another Peru-3
15 derivative expressing the AF/R1 fimbrial antigen of the *E. coli* strain RDEC-1 can be constructed in order to demonstrate safety and immunogenicity. This antigen mediates adherence to gut epithelium, causing a diarrheal disease in rabbits. The gene encoding AF/R1, carried on
20 the plasmid pW1, can be amplified by PCR, cloned into pJM6891 and integrated into the chromosome in the same manner as for CS6. The level of AF/R1 expression can be evaluated by immunoblotting. While this will not produce a vaccine candidates for humans, it can serve as a model
25 for demonstrating the expression of heterologous antigen by modified Peru-3 strains and the induction of protection from challenge by a heterologous organism.

The cloned AF/R1 gene carried on a high-copy number plasmid was also introduced to Peru-3 via
30 electrotransformation and maintained by culture in Luria-Bertani broth containing 50 µg/ml of ampicillin. Whole cell lysates of Peru-3 containing the AF/R1 sequences were analyzed for protein antigen expression by denaturing polyacrylamide gel electrophoresis and
35 immunoblotting using anti-AF/R1 polyclonal rabbit serum.

- 39 -

Immunoblots were developed using anti-rabbit IgG-alkaline phosphatase conjugate and BCIP. Expression of the AF/R1 gene was detected as production of approximately 18-kiloDalton protein. Thus AF/R1 antigen can be expressed
5 in Peru-3 for the formulation of a vaccine.

Using similar strategies, a vaccine strain expressing protective antigens of *Shigella*, such as lipopolysaccharide (LPS) and plasmid-derived invasive protein, can be made to protect against infectious
10 diarrhea caused by infective species of *Shigella*, such as *S. sonnei*.

In *S. sonnei*, there is only one serotype of LPS and it is the primary antigenic determinant in protection against this bacteria. Introduction of a plasmid clone
15 encoding the LPS operon into *E. coli* results in expression of LPS and is sufficient to confer upon *E. coli* the ability to be agglutinated by anti-*S. sonnei* LPS antibodies. The same plasmid introduced into the Peru-3 deletion mutant strain renders it agglutinatable.
20 Further analysis of the operon indicated that a 12 kilobase EcoR1/BamH1 fragment of this plasmid subcloned into pBR322 still confers the agglutination phenotype. This fragment can then be introduced to the chromosome at the *lacZ* gene of *V. cholerae* as described above.

25 Construction and safety of ST-CTA2 fusions.

ETEC causes diarrhea by colonization and production of two distinct toxins. The heat-labile toxin (LT) is nearly identical in sequence, structure and biological action to cholera toxin (CT). Therefore, production of
30 CT by Peru-3 derivatives is sufficient to induce antibodies capable of neutralizing both toxins. However, immunization with CT cannot confer protection from the ETEC heat-stable toxin (ST) which is a very small (19 amino acids) polypeptide produced by many clinical
35 isolates, some of which do not produce LT. Thus a

- 40 -

critical element in the candidate cholera/ETEC vaccine is the inclusion of ST sequences in Peru-3 in order to induce antibodies to this toxin.

A number of well defined derivatives of ST have been
5 generated that are devoid of toxin activity (SToxoids).
These derivatives are typically fragments of the toxin or substitution mutations in cysteine residues that form the three disulfide bonds of the protein. An SToxoid made up of the entire mature polypeptide with cysteine to alanine
10 mutations in residues 5 and 10 can be constructed to minimize or eliminate toxic activity. The gene encoding this SToxoid can be made entirely from complementary oligonucleotides produced with a DNA synthesizer. The synthetic gene can be flanked by unique restriction
15 endonuclease sites for subsequent subcloning into plasmid vectors.

The size of ST (19 amino acids) renders it an inherently poor immunogen. If intact ST or even small peptide fragments are coupled chemically or genetically
20 to other larger proteins (a carrier), ST becomes a much better immunogen and can induce neutralizing antibodies. The principal carrier used was the B subunit of LT or CT. Since foreign proteins fused to the cholera toxin A2 subunit (the domain of the enzymatic subunit which allows
25 the A fragment to oligomerize with the B subunit pentamer) can bind to the pentamer and form holotoxin-like complexes, these chimeric complexes are i) secreted by *V. cholerae* , ii) capable of binding the ganglioside receptor, and iii) immunoreactive.

30 The synthetic gene encoding SToxoid can be fused, in frame, to the 5' end of the gene encoding CT A2 creating an SToxoid-A2 chimera. The gene fusion construct can be integrated onto the Peru-3 chromosome as described above. When co-expressed with CT B subunit, this protein can
35 form holotoxin-like complexes devoid of both ST and CT

- 41 -

biological activity and capable of binding the ganglioside receptors. Strains expressing the SToxoid-A2 chimeric protein can be analyzed by immunoblots using anti-ST antiserum to determine if the substitution

- 5 mutations result in an antigenically related protein. The SToxoids can also be compared for toxicity in the infant mouse assay.

- The infant mouse assay is carried out as follows. 2-3 day old mice are injected intragastrically with
10 protein extracts derived from these vaccine strains (or purified ST as a control), sacrificed 3-4 hours after injection and examined for increased gut-to-body weight ratio. Candidate SToxoid-A2 chimeras demonstrating the lowest toxicity, can then be analyzed for immunogenicity
15 in rabbits.

Safety, immunogenicity and efficacy of Peru-3/AF/R1.

- Initial testing of the Peru-3 expressing AF/R1 can be done in rabbits. Bacteria can be administered orally at doses of 2×10^2 , 2×10^4 , 2×10^6 , and 2×10^8 to New
20 Zealand White rabbits. Stool samples can be collected and cultured on LB agar plates with 100 $\mu\text{g/ml}$ streptomycin to enumerate colonization and shedding of bacteria. Blood can be drawn before administration of the vaccine as well as 7, 14, 21 and 28 days following
25 administration. Sera can be prepared and analyzed for the presence of antibodies specific for AF/R1 protein via an enzyme-linked immunosorbant assay (ELISA) using purified AF/R1 bound to microtiter plates, and ability to agglutinate RDEC-1 bacteria.

- 30 Animals receiving the Peru-3/AF/R1 strain can be subsequently challenged with a pathogenic strain of RDEC-1. A challenge dose of 2×10^6 organisms can be administered orally to immunized and naive rabbits and stool samples observed for diarrhea (defined as loose,
35 wet stool soiling the rectal area and loose stool in the

- 42 -

cage bottom). Diarrhea typically occurs within 3-4 days in non-immune animals. To assay colonization, rectal swab samples are cultured on lactose MacConkey agar plates and lactose positive colonies are scored for positive reaction with anti-RDEC-1 antibodies in a slide agglutination test. Protection can be defined as both inhibition of diarrhea and bacterial colonization after day four.

Safety and immunogenicity of Peru-3/CS6 and Peru-

- 10 3/SToxoid. Initial testing of the Peru-3 strains expressing CS6 and SToxoid-A2 can be done as described above. Sera can be prepared and analyzed for the presence of antibodies specific for either CS6 or SToxoid-A2 chimeric protein via an enzyme-linked
15 immunosorbant assay (ELISA) using purified CS6 or ganglioside bound to microtiter plates. The anti-CS6 sera can also be analyzed for the presence of antibodies capable of fixing complement and lysing CS6 producing *E. coli*. In this assay, bacteria bearing CS6 are mixed with
20 serum and guinea pig complement, LB broth is added, and the bacteria are plated on LB agar. Bacteriocidal activity results in a decrease in the viable counts recovered. Finally, the anti-SToxoid-A2 sera can be tested for antibodies capable of neutralizing ST activity
25 in the infant mouse toxicity assay.

Construction of attenuated *Vibrio.cholera* expressing HIV-1 antigen as recombinant cholera holotoxoid

- An approach similar to that described above can be used to construct a *V. cholerae* vaccine strain expressing
30 antigens of the Human Immunodeficiency Virus (HIV).

- A cholera shuttle plasmid which contains a bacterial transcription unit including the promoter of the heat shock protein, *htp*, and the cholera CT-B gene was constructed. The transcription unit is flanked by the
35 DNA sequences derived from the *recA* locus of *cholera* so

- 43 -

that the CT-B gene and its promoter can be integrated into the *cholera* chromosome by the homologous recombination between the DNA sequence presence both in the *recA* locus of the *cholera* genome and on the plasmid.

- 5 The shuttle plasmid also contains a gene encoding ampicillin resistance and a gene encoding streptomycin sensitivity as the selection markers.

The HIV-1 envelope protein can be expressed as a part of recombinant *V. cholerae* holotoxoid secreted by
10 the bacteria, in the form of a "sandwich" fusion protein, in which the HIV antigen is preceded by the signal sequences of the CT-A polypeptide and followed by the CT-A2 domain. The signal sequences of the CT-A and its upstream untranslated region are required for the
15 expression and secretion of the HIV-1 antigen in the bacteria. The CT-A2 domain fused to the HIV-1 antigen is required for the fusion protein to assemble with the CT-B proteins to form a recombinant cholera holotoxoid. The plasmid described above can be modified such that a PCR
20 fragment containing the Shine-Dalgarno (SD) sequences and the signal sequences of the CT-A gene, and a unique restriction endonuclease *PmeI* site for inserting the HIV-1 antigen is inserted into its *PacI* site. The plasmid can further be modified such that a second PCR fragment
25 containing both the CT-A2 domain and the CT-B gene replaces the CT-B gene. The orientation of the DNA insertion and the junction of the PCR fragment can be confirmed by DNA sequencing.

The HIV-1 antigen used in this study is a part of
30 the HIV-1 envelope glycoprotein containing the principle neutralizing domain (PND). Previous studies demonstrate that a group of synthetic peptides derived from the PND can elicit neutralizing antibody in animals. A DNA fragment derived from HIV-LAI envelope gene including the
35 PND, but without the signal sequences and the first 120

- 44 -

amino acids, is cloned into the PmeI site of the plasmid described above which contains both the CT-A2 domain and the CT-B gene. The *in frame* fusion of HIV-1 antigen and the CT-A signal peptide and CT-A2 domain can be confirmed
5 by DNA sequencing.

To construct a genetically attenuated cholera strain that carries the HIV-1 antigen, Peru-2 is used the parental strain. The plasmid containing HIV sequences can be introduced into Peru-2 strain by mating. A
10 recombinant strain of *V.cholera* which contains deletions of *ctx* and *recA* loci and expresses a non-toxic recombinant fusion protein of HIV-1 antigen was produced and named Peru101. Southern Blot analysis can be used to confirm that Peru101 contains the DNA for HIV-1 antigen
15 and Western blot analysis can be used to demonstrate the expression of HIV-A2 fusion protein by the recombinant bacteria. An ELISA using both anti-CT-B and anti-HIV antibodies can test if the recombinant cholera holotoxoid is secreted by the bacteria.

20 Preclinical evaluation in primates of immunogenicity and protective efficacy of the oral HIV 1 vaccines using SHIV model

To test the immunogenicity of *V.cholerae* recombinant Peru101 as an oral HIV-1 prophylactic vaccine, each of
25 six adult female Rhesus monkeys (*Macaca mulatta*) can be given 2×10^6 CFU freshly prepared live bacteria in 30 ml bicarbonated water. Two additional animals in the same age and sex group can be given the same dose of Peru 2 as a control. The stool samples of the animals can be
30 analyzed two days after the vaccination to detect the multiplication of *Vibrio cholera* in the intestines by determining the colony forming unit on the LB streptomycin plates. The vaginal, rectal, salivary and serum antibodies, including IgA and IgG, that are
35 specific to HIV1 and to the CT-B can be examined biweekly

- 45 -

post vaccination. The host animal's T cell proliferation and CTL responses that are specific to the input HIV1 antigen can also be examined. One or several boosts by oral, or by intramuscular and
5 intravenous injection of purified HIV1 antigen may be necessary, depending upon the level of the initial immune responses of the vaccinated animals.

If Peru101 is able to stimulate the animals to generate anti-HIV antibodies or cell mediated HIV1
10 specific immune responses, the efficacy of Peru101 as HIV1 vaccine can be tested by challenging the animals with live SHIV-LAI stocks through vaginal infusion. The two Peru 2 animals (the monkeys who received Peru2 strain) and two of the six Peru101 animals (the monkeys
15 who received Peru101) can be challenged by 2x VI-AID₅₀ dose. Two of the other Peru101 animals will receive 10x VI-AID₅₀ and the rest of the Peru101 monkeys will receive a maximum of 50x VI-AID₅₀ dose. The peripheral blood samples can be collected every two weeks post infection
20 to determine if the animal becomes infected by detecting the viral antigen in the cultured PBMC. If the vaccine has prophylactic effect on the animals against the challenge by the SHIV carrying homologous HIV1 envelope gene, SHIV-Eli, which contains a heterologous HIV1
25 envelope, can be used to re-challenge the animals.

Use of the Live Vaccine Strains

The *V. cholerae* mutant strains Peru-1, Peru-2, Bang-1, Bang-2, Bah-1, Bah-2, Bengal -2, Bengal -3, Peru-14, and the additional mutants described above are useful as
30 sources of immunological protection against cholera and other related toxigenic diseases when used as live vaccines. Other such diseases include, but are not limited to, those induced by enterotoxigenic *E. coli* and other bacteria that produce toxins which are

- 46 -

immunologically cross-neutralizable with cholera B subunit.

When inoculated into the intestine of an experimental animal or human, mutant strains of *V. cholerae* should stimulate and induce a strong immunological response against all bacterial components that are elaborated by these strains including, but not limited to, the Ogawa and Inaba O1 LPS antigens, flagella antigens, the antigenic domains of the Tcp pili, and the outer membrane proteins. Based on published studies with other prototype cholera vaccines, both IgA and IgG classes of antibodies directed against these bacterial components will be synthesized in the inoculated animal or human and will serve to protect the animal or human against subsequent challenge with virulent strains of *V. cholerae*.

Dosage

Determination of the appropriate dosage and administration of these vaccines is performed essentially as described in Herrington et al., (1988, J. Exper. Med. 168:1487-1492). In general, such dosages are between, but are not limited to, 10^5 - 10^9 viable bacteria per dose.

Growth of Vaccine Strains

The bacteria to be used as the vaccine can be grown in a standard *V. Cholerae* laboratory media. The cells can be harvested and then lyophilized in a formulation that preserves viability (e.g., sterile skim milk or saline containing 5mM CaCl_2 and 10% weight by volume of glycerol).

- 47 -

Administration

Administration of the vaccine involves combining the contents of two envelopes or vials, one containing the lyophilized vaccine strain or combination of strains, the
5 other containing water and sufficient sodium bicarbonate or alternate buffer as to neutralize stomach acid (approximately 2 grams). The vaccine can then be swallowed by the vaccinee. Alternatively, the lyophilized vaccine can be incorporated into tablets
10 which can be coated with an acid resistant "enteric coating". Such a form of vaccine can be administered to the vaccinee in one or more (up to three) doses spaced from a few days to several weeks apart. When used as a "booster" vaccine, the vaccine can also be administered
15 to previously vaccinated individuals in one or more doses (up to three) spaced from a few days to several weeks apart. When two or more strains are being administered they may be provided together, or in individual doses 7-28 days apart.

20 Improved Killed Oral Cholera Vaccines

Preparations of improved killed oral cholera vaccines can be made from the strains described above. The experimental cholera vaccine that is currently available is comprised of approximately 10^{11} formalin and
25 heat killed *V. cholerae* cells mixed with purified cholera toxin B subunit (Black et al., Infect. Immun. 55:1116, 1987). The four strains that are used in the preparation of the bacterial component of this vaccine produce active cholera toxin which must be completely inactivated before
30 administration to the vaccinee. The new strains described above provide a vaccine that is vastly improved compared with the vaccine of Black et al. (*Supra*) for each of the reasons given below.

- 48 -

(1) Because the strains derived from, and including Peru-2, Bang-2 and Bah-2, produce only the nontoxic B subunit of the cholera toxin and not the toxic A subunit, cultures of these strains require only mild inactivation prior to administration to a vaccinee, thus avoiding the more severe denaturing treatments such as formalin or heat. The advantages of the milder treatment are that the antigens will retain a greater degree of their native configuration and as a result they will be more immunogenic. Mild methods of inactivation that avoid chemically inactivating the bacterial proteins include microwaving the organisms, treatment with another radiation source or a mild organic solvent or detergent, or the cells may be lysed by mechanical methods such as sonication or use of a French Press.

(2) In the strains Peru-3, Bang-3 and Bah-3, the *ctxB* gene has been placed under the control of the *htp* promoter. As a result, these strains synthesize large quantities of the cholera toxin B subunit (greater than 10 $\mu\text{g/ml}$ of culture) in standard laboratory medium such as LB. This facilitates purification of large amounts of the cholera B subunit and thus these strains provide a significant advantage over other strains which only produce the B subunit in small quantities under stringent growth conditions.

(3) In the preparation of existing killed cholera vaccines, a separate bacterial strain is used to produce the B toxin subunit from the strain used as the whole cell antigen. During preparation of the B subunit it is therefore necessary to purify the B subunit away from the toxic A subunit using biochemical methods. Such purification incurs the risk that small amounts of the A subunit may contaminate the preparation of the B subunit. Using the strains described above, it is possible to generate a whole cell antigen preparation from the same

- 49 -

culture used to obtain the B subunit preparation. In the first instance, purification of the B subunit is now unnecessary because the strain does not produce the A subunit, thus reducing the amount of time and
5 considerable expense involved in production of the vaccine. Secondly, there is no risk of having any contaminating A subunit in the preparations since the bacteria simply do not encode the gene for this subunit and therefore cannot produce it. The whole cell
10 preparation can therefore be used as a vaccine with minimal risk to the vaccinee.

(4) Some bacterial strains of the invention are derivatives of *V. cholerae* of the El Tor biotype and more particularly, in the case of Peru-2, Peru-3 and Peru-4,
15 they are derivatives of an isolate (C6709-Sm) which is in fact the causative agent of the current epidemic in Latin America. If there are antigens that are unique to this particular parental strain, the vaccine derivatives described above may provide generally better protection
20 against El Tor disease in Latin America and possibly other areas in the world.

Preparation of Improved Oral Killed Cholera Vaccines

An improved oral killed cholera vaccine can be prepared as follows. A minimum of two strains,
25 preferably, selected from the Ogawa serotype (e.g., Bah-3), to the Inaba serotype (e.g., Peru-3, Peru-14, or Bang-3), and the Bengal serotype (e.g., Bengal-2 or Bengal-3) can be grown in separate cultures. One of ordinary skill in the art will know how to adjust the
30 conditions, media, etc. to maximize cell growth at 37°C. For example, cultures grown under a high level of aeration in a medium such as CYE (Mekalanos et al., 1977, Infect. Immun. 16:789) or minimal medium containing glucose, i.e., AGM4 (van de Walle et al., 1990, Appl.
35 Microbiol. Biotechnol. 33:389) can be used. When growth

- 50 -

of the bacteria has reached saturation, whole cells can be recovered from the medium by centrifugation, while proteins (including the B subunit) contained within the supernatant fraction can be obtained by

- 5 ultracentrifugation or by precipitation. The cells can be inactivated using the methods of Black et al. (Infect. Immun. 55:1116, 1987) or by milder methods (e.g., microwaving, irradiation using alpha, beta or gamma rays), treatment with organic solvents such as ethanol or
10 acetone, or they may be lysed by treatment with either a detergent or by mechanical methods, such as sonication or by using a French Press. The inactivated cells can then be combined with filtered, concentrated supernatant containing bacterial proteins (including subunit B) and
15 the mixture can be suspended in a pharmaceutically acceptable solution appropriate for oral administration (e.g., sterile saline or 2% sodium bicarbonate).

- Administration The vaccine can be administered to the vaccinee as an oral saline solution which is swallowed by
20 the vaccinee several minutes after the vaccinee has ingested 2 grams of sodium bicarbonate. Alternatively, the preparation can be lyophilized and compressed into tablets which are then coated with an acid-resistant "enteric coating" prior to administration to the
25 vaccinee. The tablets can also be microencapsulated with polymers in order to facilitate uptake of the preparation by the intestinal mucosal tissue.

- Dosage A single dose of vaccine should contain approximately 10^{11} cells and approximately 100-5000 μg of
30 cholera B subunit. It is expected that the vaccinee will require approximately two or more separate doses of vaccine administered approximately two or more weeks apart.

- 51 -

Deposit

Under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure, 5 deposit of *V. cholerae* strains C6709-Sm, P27459-Sm, E7946-Sm, Bengal-2, Bengal-3, MO10, and Peru-14 have been made with the American Type Culture Collection (ATCC) of Rockville, Maryland, USA, where the deposits were given ATCC Accession Numbers ATCC 55331 (C6709-Sm); ATCC 55333 10 (P27459-Sm); ATCC 55332 (E7946-Sm); ATCC 55436 (0139, Bengal-2; ATCC 55437 (0139, Bengal-3); and ATCC 55438 (0139, MO10). Peru-14 was deposited with the ATCC June 30, 1993.

Applicant's assignee, President and Fellows of 15 Harvard College, represents that the ATCC is a depository affording permanence of the deposit and ready accessibility thereto by the public if a patent is granted. All restrictions on the availability to the public of the material so deposited will be irrevocably 20 removed upon granting of a patent. The material will be available during the pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 C.F.R. 1.14 and 35 U.S.C. § 122. The deposited material will be maintained with all the care 25 necessary to keep it viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposited material, and in any case, for a period of at least thirty (30) years after the date of deposit or for the 30 enforceable life of the patent, whichever period is longer. Applicant's assignee acknowledges its duty to replace the deposit should the depository be unable to furnish a sample when requested due to the condition of the deposit.

- 52 -

SEQUENCE LISTING**(1) GENERAL INFORMATION:**

- (i) APPLICANT: Mekalanos, John J.
- (ii) TITLE OF INVENTION: DELETION MUTANTS AS
VACCINES FOR CHOLERA
- (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Fish & Richardson
 - (B) STREET: 225 Franklin Street
 - (C) CITY: Boston
 - (D) STATE: Massachusetts
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 02110-2804
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
 - (B) COMPUTER: IBM PS/2 Model 50Z or 55SX
 - (C) OPERATING SYSTEM: MS-DOS (Version 5.0)
 - (D) SOFTWARE: WordPerfect (Version 5.1)
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Freeman, John W.
 - (B) REGISTRATION NUMBER: 29,066
 - (C) REFERENCE/DOCKET NUMBER: 00742/007001
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (617) 542-5070
 - (B) TELEFAX: (617) 542-8906
 - (C) TELEX: 200154

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:**(i) SEQUENCE CHARACTERISTICS:**

- (A) LENGTH: 17
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CCTAGTGCGC ATTATGT

17

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

SUBSTITUTE SHEET

- 53 -

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 52
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TAAACCTAGA GACAAAATGT TCCTAGTGCG CATTATGTAT GTTATGTTAA AT 52

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TAAACCTAGA GACAAAATGT TCCTAGTGCG CATTATGTGG CGCGGCAT 48

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AAACCCTAGA TTCCGCCGCC TTAGTGCGCA TTATGTATGT TATGTTAAAT 50

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

SUBSTITUTE SHEET

- 54 -

GGGCTAAAGT TAAAAGACAA ATATTTTCAG GC

32

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GGGTAGAAGT GAAACGGGGT TTACCG

26

- 55 -

What is claimed is:

1. A nontoxinogenic genetically stable mutant strain of *V. cholerae*, said strain being a genetically engineered deletion mutant lacking DNA encoding a functional *ctxA* subunit, said mutant further lacking any functional *attRS1* sequences.
2. A method of making a genetically stable mutant strain of *V. cholerae* lacking DNA encoding a functional *ctxA* subunit and further lacking any functional *attRS1* sequences, said method comprising introducing into a wild type *V. cholerae* a plasmid comprising a fragment of *V. cholerae* DNA which is mutated in its *ctxA* and *attRS1* sequences, said DNA being capable of recombining with wild type *V. cholerae* DNA inside said *V. cholerae* resulting in the generation of said mutant strain.
3. The *V. cholerae* strain of claim 1, wherein said strain is derived from a parental strain belonging to the El Tor serogroup.
4. The *V. cholerae* strain of claim 3, wherein said strain is derived from a parental strain belonging to the Inaba or Ogawa serotype.
5. The *V. cholerae* strain of claim 4, wherein said strain is Peru-2, Bang-2 or Bah-2.
6. The *V. cholerae* strain of claim 1, wherein said strain is derived from the non-01 serogroup.

- 56 -

7. The *V. cholerae* strain of claim 1, wherein said strain lacks CTX core sequences and is fully deleted for all said attRS1 sequences.

8. The *V. cholerae* strain of claim 1, wherein
5 said strain further lacks a functional *recA* gene.

9. The *V. cholerae* strain of claim 1, 7 or 8, wherein said strain further encodes a B subunit of cholera toxin.

10. The *V. cholerae* strain of claim 1, 7 or 8,
10 wherein said strain further encodes a heterologous antigen.

11. The *V. cholerae* strain of claim 10, wherein said heterologous antigen is a Shiga-like toxin or a *Shigella* lipopolysaccharide antigen, or an *E. coli*
15 fimbrial antigen or an HIV antigen.

12. The *V. cholerae* strain of claim 10, wherein the DNA sequence encoding said heterologous antigen is inserted into the *lacZ* gene of *V. cholerae*.

13. The *V. cholerae* strain of claim 9, wherein
20 said strain is Peru-3, Peru-4, Bang-3, Bah-3 or Bah-4.

14. The method of claim 2, wherein said *V. cholerae* strain is Peru-2, Bang-2 or Bah-2.

15. The method of claim 2, wherein said mutant strain lacks CTX core sequences and all attRS1 sequences.

25 16. The method of claim 2, wherein said mutant strain further lacks a functional *recA* gene.

- 57 -

17. The method of claim 2, wherein said mutant strain further encodes a heterologous antigen.

18. The method of claim 2, wherein said method further comprises introducing into the *lacZ* gene of said
5 mutant strain a fragment of DNA encoding an antigen.

19. The method of claim 18, wherein said mutant strain is Peru-5, Bang-5 or Bah-5.

20. A killed oral cholera vaccine, said vaccine comprising at least a first and a second *V. cholerae* strain suspended in a physiologically acceptable carrier, wherein each strain lacks DNA encoding a functional *ctxA* subunit, and wherein at least two of said strains are different serotypes, said *V. cholerae* being non-viable, said vaccine further comprising cholera toxin
15 B subunit which is overproduced by at least one of said serotypes of said *V. cholerae* strain.

21. The vaccine of claim 20, wherein one of said serotypes is an Ogawa serotype and another of said serotypes is an Inaba serotype.

20 22. The vaccine of claim 21, wherein said vaccine comprises Bah-3 and either Peru-3 or Bang-3 or both Peru-3 and Bang-3.

23. A nontoxinogenic genetically stable mutant strain of *V. cholerae*, said strain being a genetically
25 engineered deletion mutant lacking DNA encoding a functional *ctxA* subunit, said strain being a soft agar penetration-defective mutant.

- 58 -

24. A vaccine comprising at least two different strains of *V. cholerae* according to claim 23, one of said strains being derived from Peru and the other being derived from Bengal.

5 25. The vaccine of claim 24, wherein each of said strains is ctx⁻, att⁻, and recA⁻.

26. The vaccine of claim 23 wherein said strain is att⁻.

27. A method of making a killed *V. cholerae*
10 vaccine, said method comprising the steps of
providing at least the first and second *V. cholerae* strains of claim 20, which strains have been killed;

adding to said killed strains cholera toxin B
15 subunit produced by at least one of said strains, wherein said toxin B subunit is obtained from the medium in which said strain was propagated; and

suspending said killed strains and said toxin B subunit in a physiologically acceptable carrier.

20 28. A vaccine comprising the strain of claim 1 in a physiologically acceptable carrier.

29. The *V. cholerae* strain of claim 4, wherein said strain is Peru-14.

30. The *V. cholerae* strain of claim 6, wherein
25 said strain is of the Bengal serogroup.

31. The *V. cholerae* strain of claim 6, wherein said strain is Bengal-2 or Bengal-3.

- 59 -

32. The *V. cholerae* strain of claim 23, wherein at least 25% of the cells of said strain are capable of forming fillamentous structures of 15nM or greater under conditions of stationary phase growth.

CTX GENETIC ELEMENT

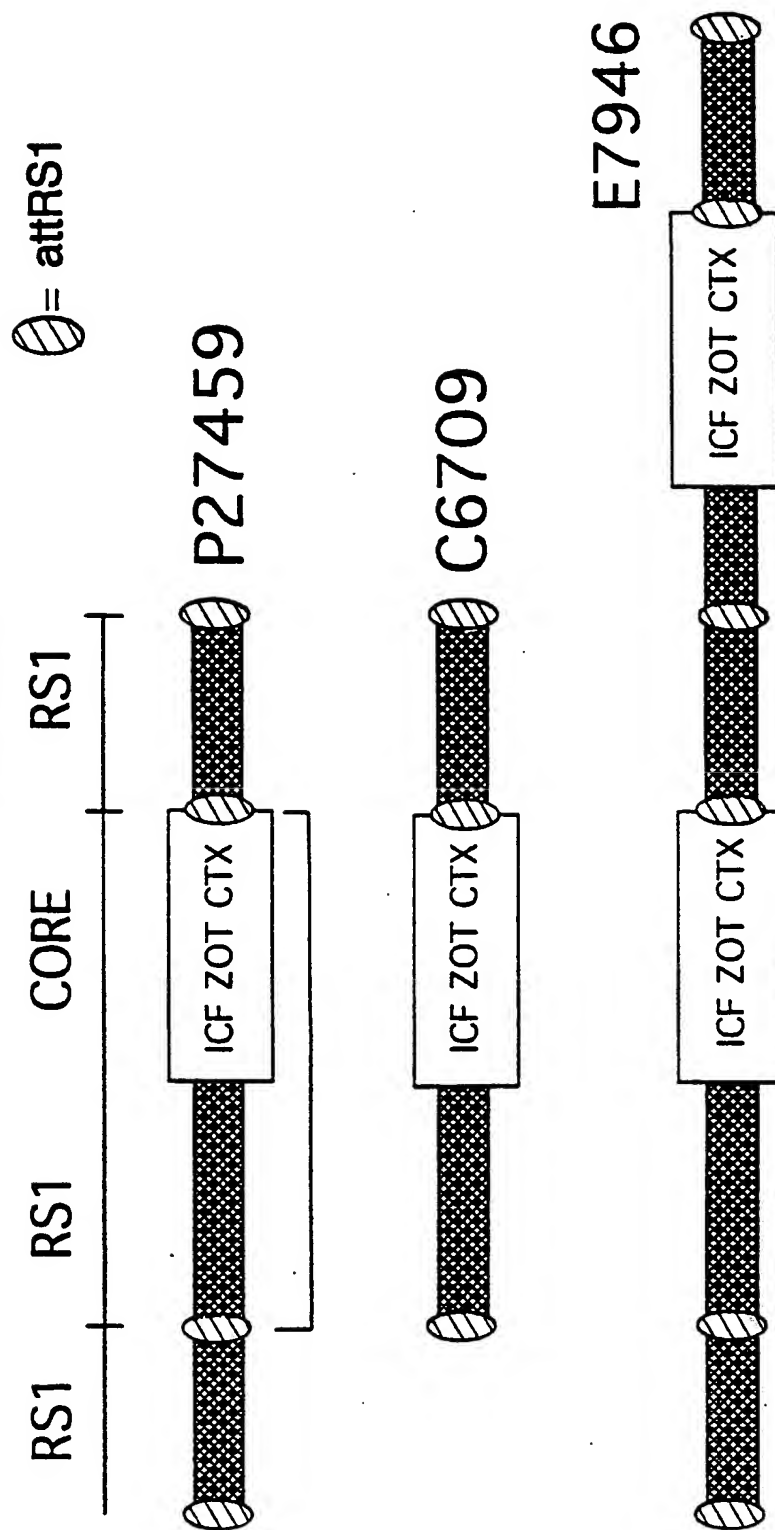


FIG. 1

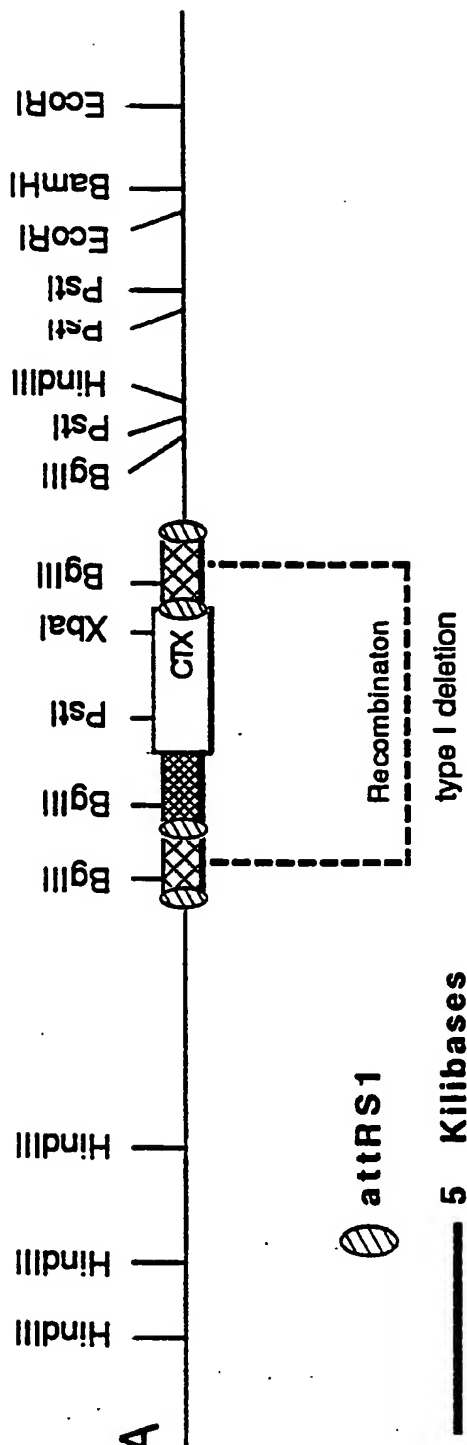


FIG. 2A

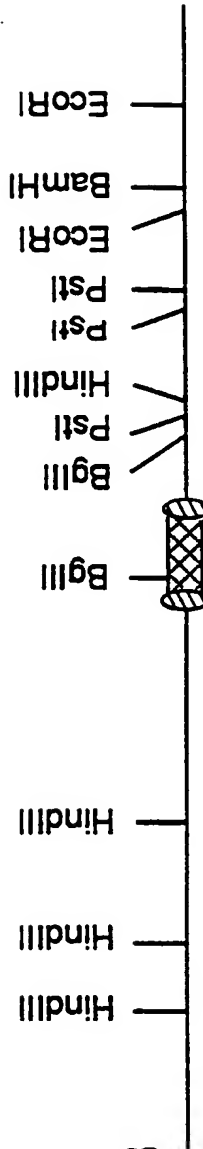
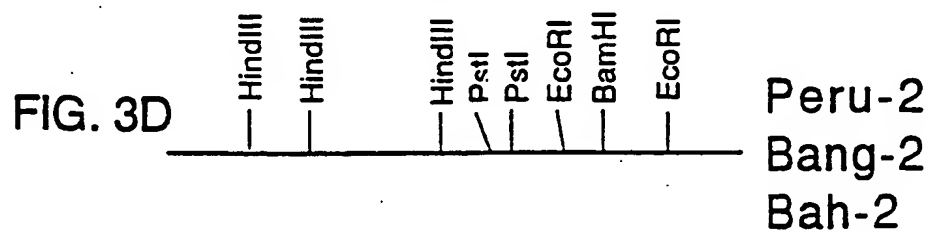
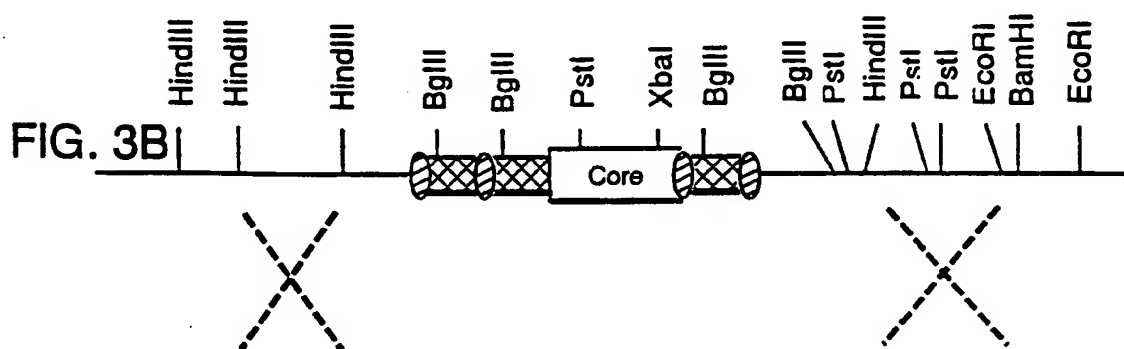
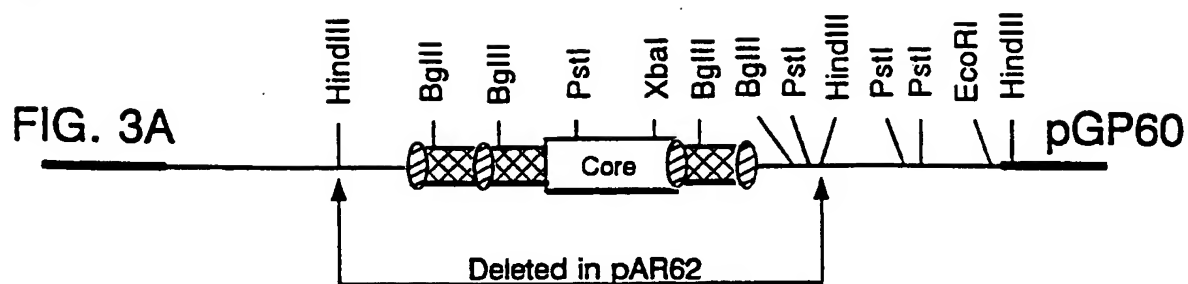


FIG. 2B

3/6

 = attRS1 sites



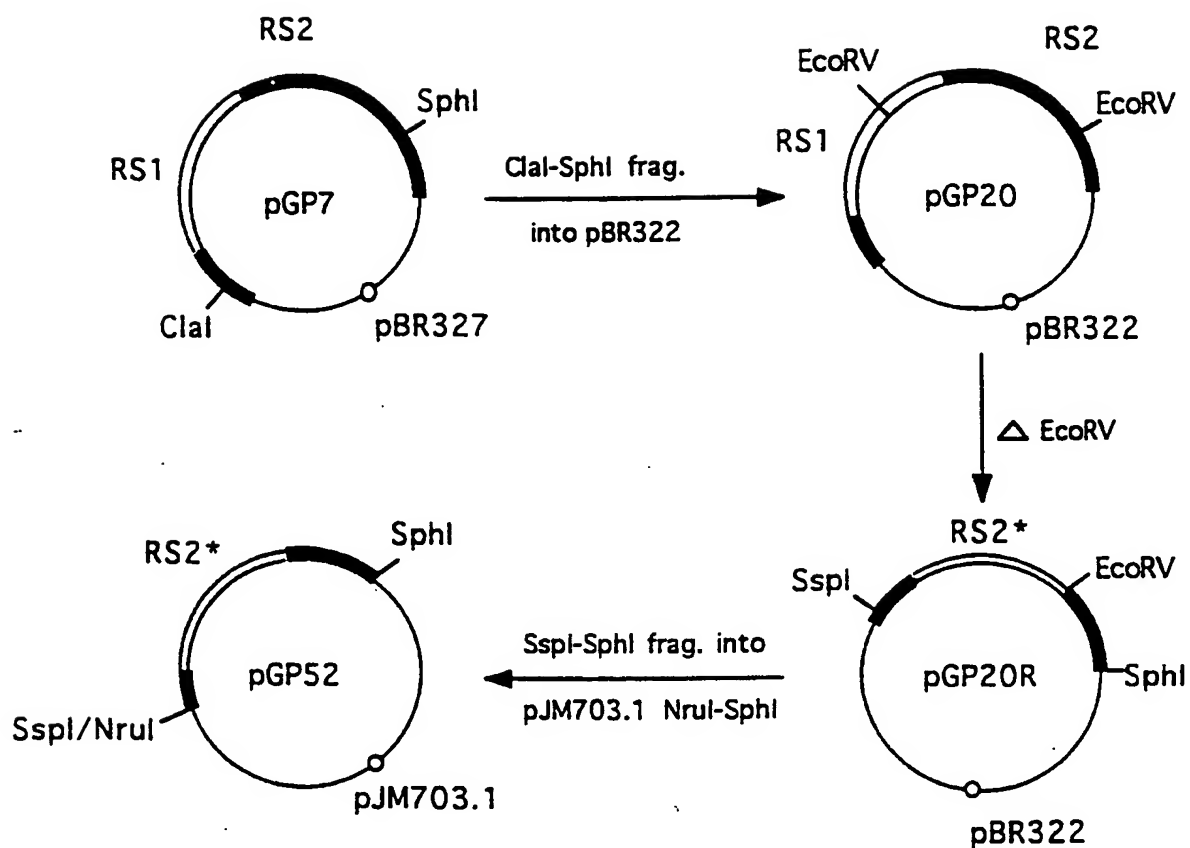


FIG. 4

5/6

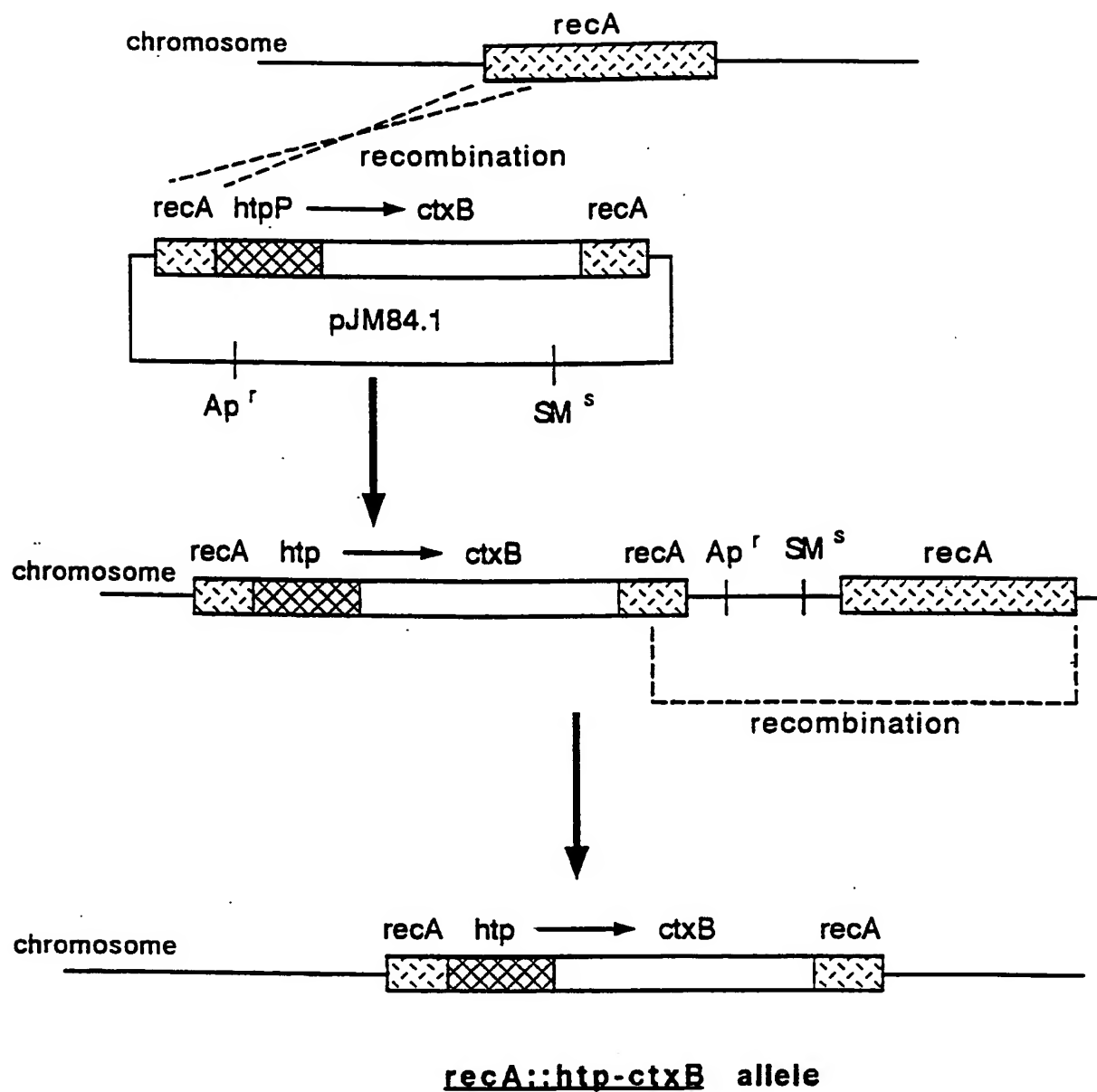
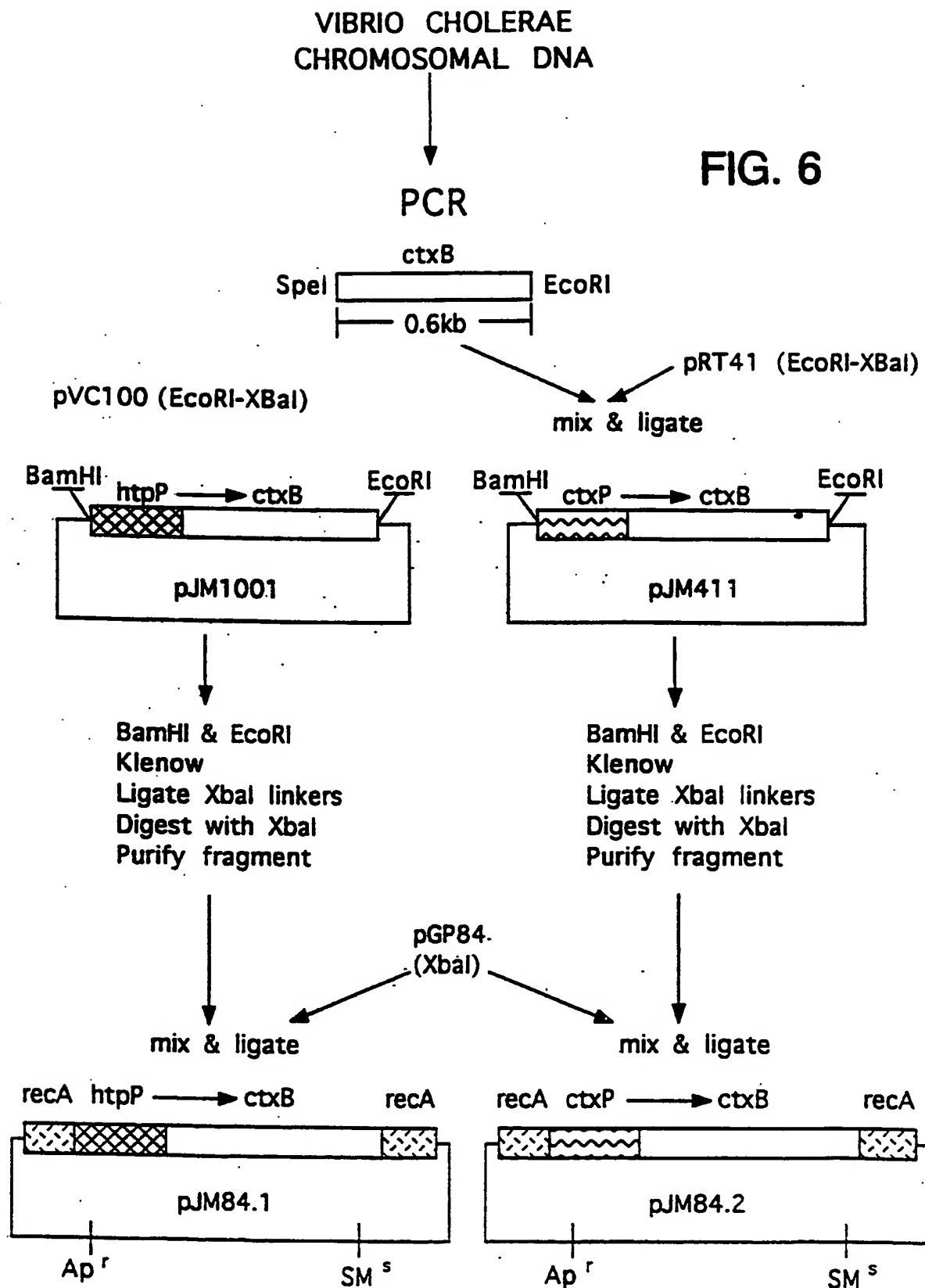


FIG. 5

SUBSTITUTE SHEET

6/6

FIG. 6



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/06270

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12N 1/21; A61K 39/106

US CL : 424/92, 93A; 435/172.1, 172.3, 909; 935/38, 65

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/92, 93A; 435/172.1, 172.3, 909; 935/38, 65

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,882,278 (MEKALANOS) 21 November 1989, see entire document.	1-32
Y	RESEARCH IN MICROBIOLOGY, Volume 141, issued 1990, G.D.N. Pearson et al, "New Attenuated Derivatives of <u>Vibrio cholerae</u> ", pages 893-899, see entire document.	1-19, 23-26, 28-32
Y	A.I. LASKIN et al, "CRITICAL REVIEWS IN MICROBIOLOGY", published May 1973, see pages 533-623.	5, 13, 14, 19, 20-22, 27

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

11 August 1993

Date of mailing of the international search report

30 AUG 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

ANTHONY C. CAPUTA

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

P. /US93/06270

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CELL, Volume 35, Number 1, issued November 1983, J.J. Mekalanos et al, "Duplication and Amplification of Toxin Genes in <u>Vibrio cholerae</u> ", pages 253-263, see entire document.	1-19, 23-26, 28-32
Y	THE LANCET, Volume 337, Number 8749, issued 04 May 1991, I.K. Wachsmuth et al, "Difference between Toxigenic <u>Vibrio cholerae</u> 01 from South America and US Gulf Coast", pages 1097-1098, see entire document.	20-22, 27
Y	BIO/TECHNOLOGY, Volume 2, issued April 1984, J.B. Kaper et al, "A Recombinant Live Oral Cholera Vaccine", pages 345-349, see entire document.	1-19, 23-26, 28-32
Y	DISSERTATION ABSTRACTS INTERNATIONAL, Volume 52, issued April 1992, G.D.N. Pearson et al, "The Cholera Toxic Element: A Site-Specific Transposon", see page 5094.	1-19, 23-26, 28-32
Y	APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, Volume 33, Number 4, issued July 1990, M. van de Walle et al, "Production of Cholera Toxin Subunit B by a Mutant Strain of <u>Vibrio cholerae</u> ", pages 389-394, see entire document.	20-22, 27
Y	JOURNAL OF CLINICAL MICROBIOLOGY, Volume 26, Number 10, issued October 1988, T. Yamamoto et al, " <u>Vibrio cholerae</u> Non-01: Production of Cell- Associated Hemagglutinins and In Vitro Adherence to Mucus Coat and Epithelial Surfaces of the Villi and Lymphoid Follicles of Human Small Intestines Treated with Formalin", pages 2018-2024, see abstract.	6, 30, 31
Y	JOURNAL OF GENERAL MICROBIOLOGY, Volume 137, Number 12, issued December 1991, W.T. Wibawan et al, "Influence of Capsular Neuramic Acid on Properties of Streptococci of Serological Group B", pages 2721-2725, see entire document.	23-26, 32

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/06270

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

BIOSIS, HEALTH PERIODICALS, DISSERTATION ABSTRACTS, MEDLINE, LIFE SCIENCE COLLECTIONS,
TOXLINE, DERWENT BIOTECHNOLOGY, EMBASE, PASCAL, APS, REGISTRY, CA
SEARCH TERMS: MEKALANOS, RECOMB?, MUTA?, ATTRS, CTXA, CHLORA?, CHOLERA?, DELET?